ROLES OF TUBEROUS SCLEROSIS COMPLEX PROTEINS IN AUTOPHAGY AND CELL DEATH

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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30th MAY 2013

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SUMMARY

Tuberous sclerosis complex 1 (TSC1) and -2 (TSC2) proteins form a functional complex to negatively regulate the mechanistic target of rapamycin (mTOR), a serine/threonine protein kinase that regulates cell proliferation, protein synthesis and autophagy. It has been demonstrated earlier that cells deficient of TSC proteins are known to have constitutively higher level of mTORC1 activity and susceptible to cell death induced by various stress factors. However, the exact function of the TSC proteins in cell stress responses has not well explored. Therefore, the main objective of this study is to investigate the roles of TSC proteins in cell death by focusing on the involvement of autophagy and JNK signaling pathway, with the following aims: 1) elucidation of the role of TSC to autophagy in response to starvation and 2) examination of the role of TSC in oxidative stress-mediated JNK signaling.

In the first part of our study, we found that TSC-null mouse embryonic fibroblasts (MEFs) were indeed more sensitive in response to various cell death stimuli, such as starvation, hypoxia and oxidative stress. The TSC2-deficient cells possessed a lower basal and inducible autophagy level, mainly due to the hyperactivation of the mTORC1 activity. Suppression of autophagy through pharmacological inhibition or genetic knockdown of Atg7 sensitised the TSC2 wild-type (TSC2WT) cells significantly, but not the TSC2-null MEFs. In contrast, ablation of mTORC1 activity via raptor knockdown or by pharmacological inhibitors in TSC2-deficient cells activated the autophagy process while simultaneously rescued the cells from starvation-mediated cell death. Notably, we

have also demonstrated that nutrients supplementation, which activated the Akt-mTORC1 signaling, enhanced cell death in TSC2-null cells but significantly reduced cell death in the TSC2WT cells. Taken together, our data demonstrate that constitutively-activated mTORC1 in TSC2-deficient cells suppresses autophagy and thus contributes to the hypersensitivity of TSC-null cells to apoptosis when stimulated with various cell death stimuli.

In the second part of our study, we systematically examined the changes of c-Jun N-terminal kinase (JNK) in the TSC-deficient cells in response to oxidative stress. We first demonstrated that TSC-null MEFs had a significantly lower level of JNK activation induced by H_2O_2 while the reconstitution of TSC2 protein expression restored the impaired JNK activation in TSC2-deficient cells. Importantly, neither the mTOR activity, nor the upstream JNK signaling kinases such as MKK4, MKK7 and ASK1 were directly involved in the defective JNK activation in the TSC2-null cells. Notably, the TSC2-deficient cells exhibited a significantly reduced activation of tyrosine phosphorylation in response to oxidative stress and one key finding from our study was that the TSC may be involved in stabilizing the JNK phosphatase, MAPK phosphatase (MKP-1) activity. Finally, the impairment of JNK signaling in TSC2-deficient cells appears to promote necrotic cell death in these cells. Thus, our data demonstrates a novel function of TSC in mediating the cell death through JNK-MKP1 signaling in response to oxidative stress. In summary, data from our study demonstrate that TSC protein plays vital roles in the regulation of autophagy and cell death in response to stress.

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LIST OF ABBREVIATIONS

4EBP1	eukaryotic translation-initiation factor 4E-binding protein
AgRP	agouti-related peptide
AIF	apoptosis-inducing factor
AMPK	adenosine monophosphate-activated protein kinase
AP-1	activator protein 1
APAF-1	apoptosis protease-activating factor-1
ARE	AU-rich element
ASK1	apoptosis signal regulating kinases 1
Atg	autophagy related
ATM	Ataxia-telangiectasia mutated
Bad	Bcl-2-associated death promoter protein
Bcl	B-cell lymphoma
Bcl-xL	Bcl-extra large
Beclin 1	Bcl-2-interacting protein
BH	Bcl-2 homology
Bif-1	Bax-interacting factor 1
BNIP3	Bcl-2/adenovirus E1B 19 kD protein-interacting protein 3
BRCA1	breast cancer 1
BSA	bovine serum albumin
C/EBPβ	CCAAT/enhancer binding protein beta
cAMP	cyclic adenosine monophosphate
Caspase	cysteinyl aspartate-specific protease
CD	cluster of differentiation
CD95L	cluster of differentiation 95 ligand
cDNA	complementary DNA
cFLIP	cellular form of the caspase-8 inhibitor FLIP
cIAP1	cellular inhibitor of apoptosis 1
CMA	chaperone-mediated autophagy
CO_2	carbon dioxide
COP	coatomer protein
CQ	chloroquine diphosphate
CREB	cAMP response element-binding protein
C-terminus	carboxy-terminus
CUL3	cullin3
CYLD	Cylindromatosis
cyt *c*	cytochrome *c*
DAP1	death-associated protein-1
DAPk	death-associated protein kinase
DEPTOR	DEP domain containing mTOR-interacting protein
DIABLO	direct inhibitor of apoptosis-binding protein with low pi
DIF	differentiation factor
DISC	death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid

dNTP	deoxyribonucleotide triphosphate
DR	death receptor
DRAM	damage-regulated autophagy modulator
Drp1	dynamin-related protein 1
EBSS	Earles's balanced salt solution
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
eIF4	eukaryotic translation initiation factor 4
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
ESCRT	endosomal sorting complex required for transport
ETC	electron transport chain
EV	empty vector
FADD	Fas-associated death domain
FAK	focal adhesion kinase
Fas	apoptosis stimulating fragment
FasL	Fas ligand
FBS	fetal bovine serum
FFA	free fatty acids
FIP200	FAK-family-interacting protein of 200kD
FLICE	FADD-like IL-1β-converting enzyme
FLIP	FLICE-inhibitory protein
FRB	FKBP12-rapamycin binding
GAP	GTPase Activating Protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptors
GSK3β	glycogen synthase kinase 3β
GTP	Guanosine triposphate
H_2O_2	hydrogen peroxide
HDAC6	histone deacetylase 6
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIF1	hypoxia-inducible factor-1
HIV	human immunodeficiency virus
HMGB1	high-mobility group box 1
HOPS	homotypic fusion and vacuole protein sorting
Hsp70	heat-shock protein 70
HSV-1	herpes simplex virus type 1
hVps34	human vacuolar protein sorting protein
IFN	Interferon
IGF-1	insulin-like growth factor-1
IKK	IκB kinase
IL	Interleukin
IMS	intermembrane space
IP	immunoprecipitation
IRE1α	inositol requiring 1α

IRS-1	insulin receptor substrate-1
IκB	NF-κB inhibitory protein
JNK	c-Jun N-terminal kinases
JNKK2	JNK kinase/MKK7
KLHDC10	Kelch domain containing 10
LAM	Lymphangioleiomyomatosis
LAMP-2	lysosomal-associated membrane protein 2
LC3	microtubule-associated protein light chain 3
LE	longer exposure
LIR	LC3-interacting region
Lys63-Ub	lysine63-linked polyubiquitination
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase/MEK/MKK
MAPKKK	MAPKK kinase/MEK kinase/MAP3K
Mcl-1	myeloid cell leukemia sequence-1
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
miR	micro RNA
MKPs	map kinase phosphatases
MLK	mixed lineage kinase
mLST8	mammalian lethal with sec-13 protein 8
mn-SOD	mitochondrial superoxide dismutase
MOMP	mitochondrial outer membrane permeabilization
mRNA	messenger RNA
mSin1	mammalian stress-activated map kinase-interacting protein 1
mTORC	mechanistic/mamalian target of rapamycin complex
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
N_2	Nitrogen
NaCl	sodium chloride
NaN_3	sodium azide
NBR1	neighbour of BRCA1 gene 1
Nec-1	necrostatin-1
NF-IL6	nuclear factor for the IL-6 gene
NF-κB	nuclear factor kappa B
NLR	NOD-like receptors
NOD	nucleotide oligomerization domain
Nox1	NADPH oxidase 1
NP-40	Nonidet P-40
NS	non-specific
O^{2-}	superoxide anion
OATL1	ornithine aminotransferase-like 1
p-	phosphorylation of
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction

PDK1	phosphatidylinositol-dependent kinase 1
PE	Phosphatidylethanolamine
PGAM5	phosphoglycerate mutase 5
PGC1α	PPAR-γ coactivator 1α
PI	propidium iodide
PI3K	phosphoinositide-3 kinase
PIKK	PI3K-related protein kinase
PINK1	PTEN-induced putative kinase 1
PK	protein kinase
Pol 1	Polymerase I
PP5	protein phosphatase 5
PPAR-γ	peroxisome proliferator-activated receptor gamma
PRAS40	proline-rich Akt substrate of 40 kD
Protor	protein observed with rictor-1
PtdIns(4,5)P2	phosphatidylinositol-4,5-bisphosphate
PtdIns3P	Phosphatidylinositol 3-phosphate
PTEN	Phosphatase and tensin homolog
PTPs	protein tyrosine phosphatases
PVDF	polyvinylidene difluoride
qPCR	quantitative PCR
RAB7A	Ras-related GTP-binding protein 7A
Rag	Ras-related GTPase
Raptor	regulatory associated protein of mTOR
RB1CC1	Retinoblastoma1-inducible coiled-coil 1
REDD1	regulation of DNA damage response 1
RFK	riboflavin kinase
Rheb	Ras homolog enriched in brain
Rictor	rapamycin-insensitive companion of mTOR
RIP	receptor interacting protein
RNA	ribonucleic acid
Romo1	ROS modulator 1
ROS	reactive oxygen species
RSK	p90 ribosomal S6 kinase
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
Rubicon	RUN domain and cys-rich domain containing Beclin 1 interacting protein
S.O.C	Super Optimal broth with Catabolite repression
S6K	p70 S6 kinase
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SE	shorter exposure
SEGA	subependymal giant cell astrocytomas
SGK1	serum- and glucocorticoid-induced protein kinase 1
siRNA	short interference RNA

SMAC	second mitochondria-derived activator of caspase
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNP	sodium nitroprusside
SREBP	sterol regulatory element binding proteins
SRF	serum response factor
STK11	serine/threonine kinase 11
TAK1	TGF-β-activated kinase 1
tBid	truncated BH3 interacting domain death agonist
TBST	Tris Buffered Saline with Tween 20
TCA	tricarboxylic acid
TE	Tris and EDTA
TECPR1	tectonin beta-propeller repeat containing 1
Tel2	telomere maintenance 2
TFEB	transcription factor EB
TGF-β	transforming growth factor beta
Th	T helper
TIF-1A	transcriptional intermendiatery factor 1 Alpha
TLR	Toll-like receptors
TM	turn motif
TNF	tumour necrosis factor
TNFR	TNF receptor
TOR	target of rapamycin
TRADD	TNFα receptor-associated death domain
TRAF	TNFα receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAILR	TRAIL receptor
TRIF	Toll/IL-1 receptor domain-containing adaptor inducing IFN-β
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
TSC	tuberous sclerosis complex
Tti1	Tel two interacting protein 1
ULK	Unc-51-like kinase
UV	Ultraviolet
UVRAG	UV irradiation resistance-associated gene
WIPI2	WD repeat domain phosphoinositide-interacting protein 2
WT	wild type
XIAP	X-linked inhibitor of apoptosis protein
YY 1	Ying-Yang 1
zVAD	carbobenzoxy-Val-Ala-Asp-(OMe)- fluoromethylketone

LIST OF PUBLICATIONS

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Zhou J, **Ng S**, Huang Q, Wu YT, Li Z, Yao SQ, and Shen HM (2013). AMPK mediates a pro-survival autophagy downstream of PARP-1 activation in response to DNA alkylating agents. **FEBS Lett**, 2013 Jan;587 (2):170-7.

TSC2 protein promotes oxidative stress-mediated JNK activation via disruption of MKP-1 function. (Manuscript in preparation)

CHAPTER ONE

INTRODUCTION

1.1 Autophagy

1.1.1 Overview of Autophagy

Autophagy is a major intracellular degradation system in which cytoplasmic materials are delivered to the lysosome for degradation, and subsequently recycled to form new building blocks for cellular renewal and maintaining homeostasis (Mizushima and Komatsu, 2011). In mammalian cells, there are three classes of autophagy, namely macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy (referred as autophagy thereafter in this thesis) involves the formation of an omegasome and an isolation membrane that sequesters a small portion of the cytoplasm to form the double-membrane vesicles known as autophagosomes. This autophagosome then fuses with lysosome to form autolysosome for cellular degradation. Meanwhile, microautophagy takes place by the engulfment of components in cytoplasm through the inward invagination of the lysosomal membrane. In CMA, substrate proteins are stationed directly across the lysosomal membrane (Mizushima and Komatsu, 2011). The molecular machinery of autophagy will be discussed in detail below.

1.1.2 Autophagic process and its machinery

Autophagy is a dynamic, catabolic process that is evolutionary conserved throughout the unicellular and multicellular eukaryotes (Lebovitz et al., 2012; Yang and Klionsky, 2010b). The most common stimulus of autophagy is nutrients starvation, such as amino acid starvation in mammalian cells and nitrogen starvation in yeasts as well as in plants (Mizushima, 2007). Meanwhile,

rapamycin and other mechanistic target of rapamycin complex (mTOR) kinase inhibitors have been used to upregulate autophagy under nutrient-rich conditions (Mizushima et al., 2011). The autophagy machinery studies was first discovered in yeasts *Saccharomyces cerevisiae* (Tsukada and Ohsumi, 1993). In mammalian cells, the mechanistic process of autophagy can be divided into four stages, which are i) induction, ii) nucleation, iii) elongation/expansion and finally, iv) maturation of the autophagosome to form autolysosome (Lebovitz et al., 2012). Figure 1.1 illustrates the autophagic process and its machinery.

1.1.2.1 Induction

The first significant event in autophagy is the induction or initiation of the membrane of an autophagosome to form the phagophore or isolation membrane. This double-membrane structure formed has the ability to grow and expand through selective engulfment of proteins and organelles while extending its membrane (Tooze and Yoshimori, 2010). Though it still remains interesting to study the origin of this membrane, recent evidence has proposed that endoplasmic reticulum (ER) (Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009) and mitochondria (Hailey et al., 2010) are the sources.

The induction process is initiated by the activation of Unc-51-like kinases (ULK) complex, comprising of ULK1 and ULK2, Atg13, the scaffold focal adhesion kinase (FAK)-family-interacting protein of 200 kD (FIP200, also known as Retinoblastoma1-inducible coiled-coil 1, RB1CC1) and Atg101 (an Atg13-binding protein) (Chen and Klionsky, 2011). ULK is the mammalian homolog of Atg1 gene in yeast, and is the only known protein kinase among all Atg proteins

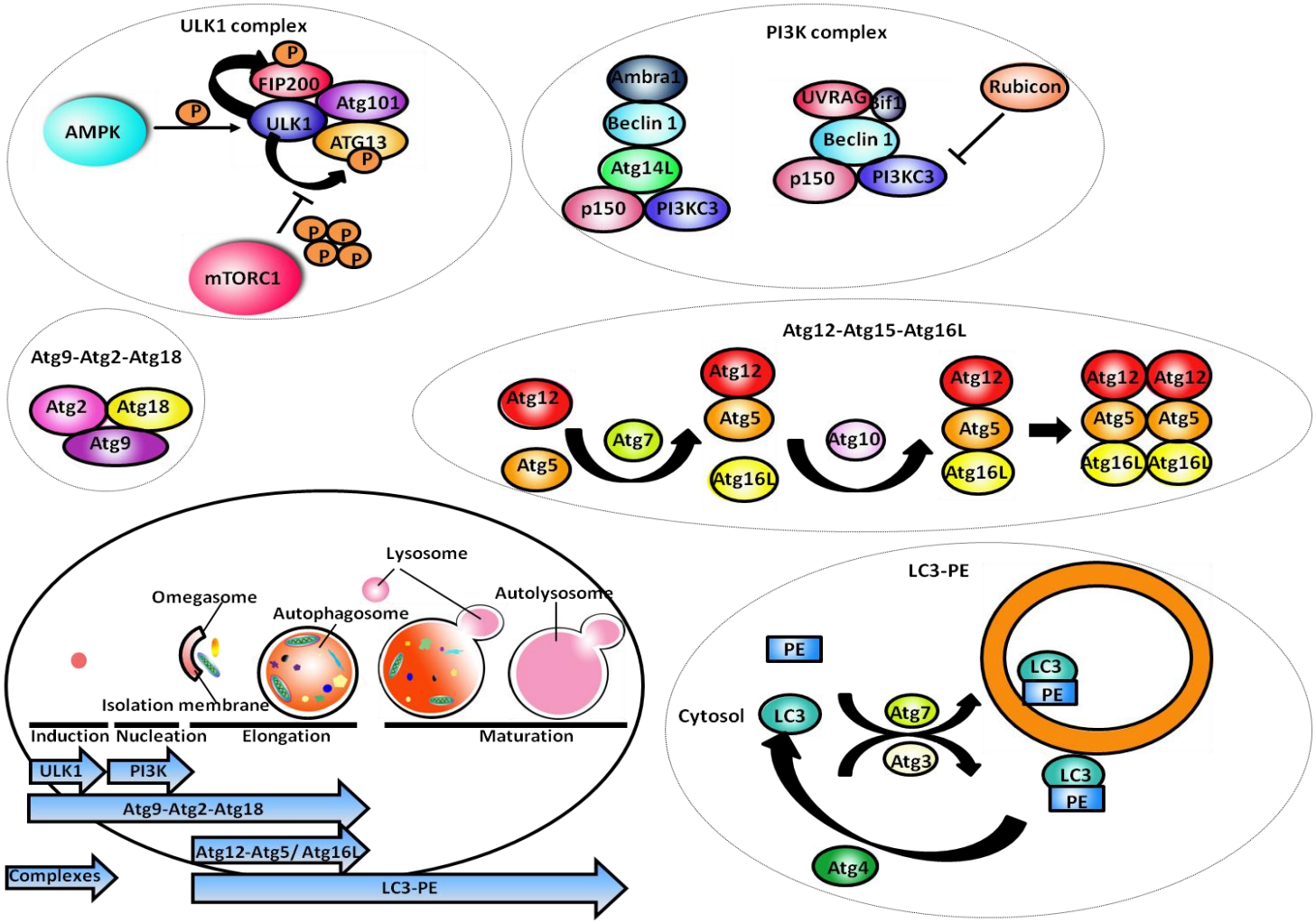


Figure 1.1 The autophagy machinery.

(Mizushima et al., 2011). The major homolog of Atg1 could be ULK1 (Chan et al., 2007) as ULK2 could have a redundant function (Kundu et al., 2008; Mizushima et al., 2011).

In contrast to yeasts' Atg1 complex disassembly upon starvation, the ULK1-Atg13-FIP200-Atg101 complex is constitutively formed in the cytosol of mammalian cells but is inactivated by mTORC1 (Hosokawa et al., 2009; Jung et al., 2009). mTORC1 is a major cell regulator that will be described later in this chapter (please refer to Section 1.3). During nutrients availability, mTORC1 phosphorylates ULK1, ULK2 as well as Atg13 and there is a direct interaction of raptor (a regulatory subunit of mTORC1) to ULK1 (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). Under starvation conditions, the mTORC1 is inactivated and dissociates from this complex, thereby leading to partial dephosphorylation and activation of the ULK1 complex (Hosokawa et al., 2009). The activated ULK1 phosphorylates and thereby activates FIP200, causing a stable ULK1-Atg13-FIP200-Atg101 complex that initiates the induction of autophagy machinery (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). The ULK complex is also additionally regulated by cAMP-dependent protein kinase A (Stephan et al., 2009) and the more recent discovery of adenosine monophosphate-activated protein kinase (AMPK) signaling (Egan et al., 2011; Kim et al., 2011; Lee et al., 2010c; Shang et al., 2011).

1.1.2.2 Nucleation

In autophagy, the activation of Class III phosphoinositide 3-kinases (PI3K) complex is the key positive regulator for the nucleation step of

autophagosome formation. In mammalian cells there are three classes of PI3K. Depending on substrate specificity preference and sequence homology, PI3Ks are categorised into respective three classes of class I, II and III that regulate diverse roles of cellular processes (Domin and Waterfield, 1997; Vanhaesebroeck et al., 2012). Class I PI3K has substrate specificity to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to form PtdIns(3,4,5)P_3, while Class II PI3K utilises PtdIns and PtdIns4P as a substrate to produce PtdIns(3,4)P_2 and convert PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Meanwhile, Class III PI3K (PI3KC3) contains the catalytic member human vacuolar protein sorting protein (hVps34) that specifically uses PtdIns as a substrate to generate PtdIns3P for nascent autophagosome formation (Mizushima et al., 2011; Vanhaesebroeck et al., 2010).

The Class III PI3K complex includes hVps34, Beclin 1 (a homolog of yeast Atg6) (Furuya et al., 2005; Zeng et al., 2006), p150 (Petiot et al., 2000), Atg14-like protein (Atg14L or also known as Barkor) (Matsunaga et al., 2009; Zhong et al., 2009), ultraviolet irradiation resistance-associated gene (UVRAG) (Liang et al., 2008), Ambra1 (Fimia et al., 2007) and Bax-interacting factor 1 (Bif-1) (Takahashi et al., 2007). There are two complexes formed for Class III PI3K that positively regulates autophagosome formation, which are i) Beclin 1-Ambra1-Atg14L-p150-PI3KC3 or 2) UVRAG-Bif1-Beclin 1-p150-PI3KC3 (Janku et al., 2011). In contrast, the RUN domain and cysteine-rich domain containing Beclin 1 interacting protein (Rubicon) interacts with UVRAG–Beclin 1–hVps34–p150 complex to inhibit autophagosome maturation by reducing hVps34 activity (Matsunaga et al., 2009; Zhong et al., 2009).

Class I and III PI3K are known to play negative and positive roles of autophagy, respectively (Baehrecke, 2005). However, one recent report has shown that Class IA PI3K p110-beta subunit is a positive regulator of autophagy by affiliating with the autophagy-promoting Vps34–Vps15–Beclin 1–Atg14L complex to promote the generation of cellular PtdIns3P (Dou et al., 2010), which is required for the generation of autophagosome (Noda et al., 2010).

In addition to the functions of PI3K, the Atg9-Atg2-Atg18 cycling complex is also thought to deliver lipids from source to growing autophagosome (Simonsen and Tooze, 2009). Atg9 is an interesting protein among all other Atgs proteins needed for autophagosome formation because it is the only known transmembrane protein that spans the membrane six times (Noda et al., 2000). However the functions of Atg9 has yet to be fully understood (Mizushima et al., 2011).

1.1.2.3 Elongation/expansion

The isolation membrane extends to sequester the targeted cytosolic components and organelles and finally closes to form the structure termed as autophagosome (Tooze and Yoshimori, 2010). For the formation of autophagosome, the PtdIns3P-containing membranes recruit two distinct ubiquitin-like protein conjugation systems: 1) Atg12-Atg5-Atg16L system, and 2) the microtubule-associated protein light chain 3 (LC3, is the mammalian Atg8 homolog)-phosphatidylethanolamine (PE) conjugation system. Atg7 and Atg10 (which are ubiquitin-activating enzymes, E1- and E2-like enzymes, respectively) are required to catalyse the conjugation of Atg12 to Atg5. This Atg12-Atg5

conjugate then associates with Atg16L (E3-like ligase enzyme) through oligomerization to form a large multimeric Atg16L complex. On the other hand, Atg7 and another E2-like enzyme, Atg3 mediate the ubiquitin-like conjugation of LC3-I to membrane lipid, PE. Following this step, the lipidated form of LC3 (termed as LC3-II) is generated (Mizushima et al., 2011). The LC3-I is soluble and present in the cytosol, while LC3-II is bound specifically to the membrane. LC3-II attaches to both faces of isolation membrane, but will be delipidated from the outer membrane to be recycled by Atg4, a cysteine protease that cleaves the C-terminus of LC3-II to generate cytosolic LC3-I with a C-terminal glycine residue (Mizushima et al., 2011). Meanwhile, the LC3-II in the inner membrane will be degraded together during the fusion of autophagosome to a late endosome or lysosome by lysosomal proteases (Kimura et al., 2009).

1.1.2.4 Autophagosome maturation and degradation

In the final stage of autophagy, autophagosome fuses with late endosome and lysosome to form autolysosome, a process involving rather complicated mechanisms and membrane trafficking proteins such as lysosomal-associated membrane protein 2 (LAMP2) (Fortunato et al., 2009) and the small GTPase Ras-related GTP-binding protein 7A (RAB7A) (Jager et al., 2004). These proteins mediate the docking and fusion of autophagosomes with lysosomes to form autolysosomes (Jager et al., 2004). UVRAG is also involved in controlling autophagosome maturation by activating RAB7A (Liang et al., 2008).

Presently, numerous studies have also been performed to uncover the mechanisms underlying the autophagosome maturation process. These studies

have defined other essential players such as the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Abeliovich et al., 1999; Darsow et al., 1997; Fraldi et al., 2010; Ishihara et al., 2001; Moreau et al., 2011; Nair et al., 2011), endosomal coatomer protein (COP) (Ishihara et al., 2001; Razi et al., 2009), endosomal sorting complex required for transport (ESCRT) III complex (Lee et al., 2007d; Rusten et al., 2007), homotypic fusion and vacuole protein sorting (HOPS) complex (Nickerson et al., 2009), and heat-shock protein 70 (Hsp70) chaperone (Leu et al., 2009).

More recently, the discovery of histone deacetylase 6 (HDAC6) (Lee et al., 2010d), ornithine aminotransferase-like 1 (OATL1) (Itoh et al., 2011) and tectonin beta-propeller repeat containing 1 (TECPR1) (Chen et al., 2012) has contributed more in understanding the underlying mechanism involving the fusion between autophagosome and lysosome.

1.1.3 Biological functions of autophagy

Notably, autophagy essentially plays numerous functional roles for maintaining proper cellular functions, such as in energy recycling for cell survival and in cellular degradation (Mizushima, 2007). However, autophagy regulation seems to be rather complex, as it may be beneficial to the cells as well as detrimental, such that observed in cancer development, which will be discussed in the following section. The roles of autophagy in cell survival and cell death will be further discussed in Section 1.2 in this chapter.

1.1.3.1 Autophagy in maintaining energy homeostasis

Notably, the pro-survival function of autophagy has been observed in

numerous models. One important pro-survival role of autophagy is through the recycling of cellular products components to continuously maintain the amino acid level. This is especially important during starvation as an adaptive response to support the survival of cells under stressed conditions (Mizushima, 2007). Autophagy is required for survival, at least in maintaining the amino acid pool as knockout of *Atg3* (Sou et al., 2008), *Atg5* (Kuma et al., 2004), and *Atg7* (Komatsu et al., 2005) in mice ensues in neonatal lethality. Previously, it has also been shown that autophagy is essential to maintain survival during starvation conditions in *Saccharomyces* cerevisiae (Tsukada and Ohsumi, 1993), *Dictyostelium discoideum* (Otto et al., 2003), *Drosophila melanogaster* (Scott et al., 2004), *Caenorhabditis elegans* (Kang et al., 2007), *Spodoptera litura* (Wu et al., 2011), zebrafish (Yabu et al., 2012) and also in plants (Honig et al., 2012). Through the tricarboxylic acid (TCA) cycle, amino acids can be utilised as an energy source (Newsholme et al., 1985).

Autophagy is also found to be involved in regulating other types of nutrients such as lipids. Lipophagy, a process for the degradation of liver lipid droplets by autophagy, contributes to the production of free fatty acids that are oxidised in the mitochondria (Singh et al., 2009). This relatively new type of autophagy, named as macrolipophagy, is constitutively active at basal levels in most cell types that functions in controlling the size and number of lipid droplets under basal conditions (Cuervo and Macian, 2012; Singh et al., 2009). During conditions such as energy scarcity or to prevent mass accumulation of lipids, macrolipophagy is activated (Singh et al., 2009). It is interesting to note that the

stimulatory effects of lipids such as free fatty acids (FFA) on autophagy in the hypothalamic neurons triggers the secretion of agouti-related peptide (AgRP) to elicit restoration of cellular energetic balance (Kaushik et al., 2011).

In addition to amino acids and lipids, studies have showed that autophagy is also essential in glycogen breakdown to maintain cellular energy levels (Kotoulas et al., 2006; Raben et al., 2008). Nevertheless, the contributions of autophagy in glucose production as compared to the extent of the classical cytosolic degradation of glycogen will require more investigations.

1.1.3.2 Autophagy in cellular degradation

Autophagy is a constitutively active process that functions at low levels under cell basal conditions (Mizushima and Komatsu, 2011). Autophagy clears unwanted proteins and cellular organelles to maintain the quality and condition of cells through the constitutive turnover of cytoplasmic contents (Mizushima, 2007). Basal autophagy is essential to maintain the tissue and cellular homeostasis. For examples, hepatomegaly and hepatic failure were seen in liver specific *Atg7*$^{-/-}$ mice (Komatsu et al., 2005) while neurodegeneration along with progressive motor deficits were observed in neural cell-specific *Atg5* and *Atg7* knockout mice (Hara et al., 2006; Komatsu et al., 2006). However, it is possible that only a small amount of autophagy is adequate for quality control as induced autophagy is not observed in the brain during starvation (Mizushima et al., 2004; Nixon et al., 2005).

Multiple health complications have been linked to defects in cellular degradation processes. Most neurodegenerative diseases are characterised by the

formation of protein inclusions inside neurons. One important example is neurodegenerative diseases, as an incremental amount of autophagic vacuoles and protein aggregates have been demonstrated in Alzheimer's diseases (Lee et al., 2010b; Nixon et al., 2008**),** Parkinson's disease (Anglade et al., 1997), amyotrophic lateral sclerosis (Kabuta et al., 2006), and Huntington's disease (Petersen et al., 2001; Ravikumar et al., 2002). More recently, in an axotomy model, autophagy is also seen to be cytoprotective in retinal ganglion cells as autophagy increases the number of surviving cell (Rodriguez-Muela et al., 2012). Meanwhile, unnecessary organelles such as peroxisomes is selectively degraded through microautophagy in yeasts (Sakai et al., 1998) and macroautophagy in mammals (Iwata et al., 2006); as well as mitochondrial degradation through mitophagy (Kim et al., 2007a). Recently, in *C. elegans*, paternal mitochondria are found to be degraded by fertilization-induced autophagy (Al Rawi et al., 2011; Sato and Sato, 2011). Autophagy is also implicated in cardiomyopathy (Kashio et al., 1991) and metabolic diseases such as diabetes and obesity as reviewed recently (Rubinsztein et al., 2012).

In addition, the selective degradation is also mediated by autophagy. Interactions of cellular cargos with a molecular tag (for example polyubiquitin), adaptor proteins [such as p62 and neighbour of breast cancer 1 (BRCA1) gene 1, (NBR1)] and LC3 further target them to autophagosomes (Levine et al., 2011). Both p62 and NBR1 proteins have been identified as a LC3-interacting proteins, depending on LC3-interacting region (LIR) and ubiquitin-binding protein that are selectively trapped by LC3 and degraded in the autophagosome (Bjorkoy et al.,

2005; Komatsu et al., 2007; Lamark et al., 2009; Pankiv et al., 2007). A long term inhibition of autophagy causes p62 and NBR1 accumulation and subsequently compromises the ubiquitin-proteasome degradation system (Korolchuk et al., 2009; Lamark et al., 2009).

Autophagy also removes damaged organelles, including mitochondria and ER. Mitophagy, a process whereby the mitochondria is selectively removed by autophagy during the loss of mitochondrial potential (Wang and Klionsky, 2011). For example, specific autophagy receptor such as Bcl-2/adenovirus E1B 19 kD protein-interacting protein 3-like (BNIP3L or also known as Nix) mediates mitochondria elimination during reticulocyte maturation (Schweers et al., 2007). The ubiquitination process is also important for organelle removal, such as those conferred by Parkin, an E3 ubiquitin ligase. Parkin localizes at depolarised mitochondria to induce mitophagy, along with phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) (Kim et al., 2008b; Vives-Bauza et al., 2010). Interestingly, PINK1 also interacts with Beclin 1 under basal and starvation conditions to promote autophagy (Michiorri et al., 2010).

1.1.3.3 Autophagy in cancer

While the involvement of autophagy in cancer has been extensively studied, its exact role in cancer development remains to be fully elucidated. The common understanding is that autophagy plays a dual role in cancer: autophagy favors a tumour suppressive role in the early stage of carcinogenesis, while it also protects cells during cellular and metabolic stress that favors tumour progression (Mathew et al., 2007). Autophagy acts as an intracellular cell quality controller

that prevents malignant transformation and cancer initiation, while paradoxically, its pro-survival role during stressed conditions enables the established tumours to adapt and survive (Mathew et al., 2007). Thus, the development of cancer involves a complicated processes with multiple stages (Hanahan and Weinberg, 2011). Moreover, in part of its dual role in cancer, the strategy for modulating autophagy in cancer treatments increases in its complexity (Dalby et al., 2010). Figure 1.2 briefly illustrates the roles of autophagy in tumourigenesis.

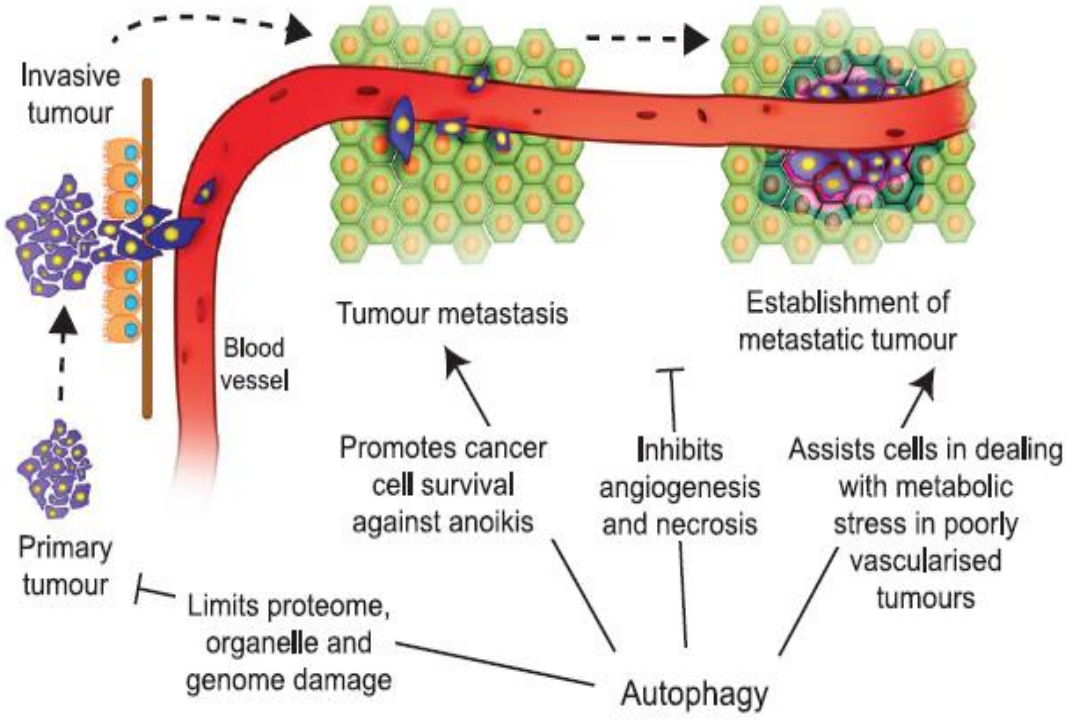


Figure 1.2 Autophagy has multiple roles in tumourigenesis, depending on the different stages of tumour development. Adapted from Liu and Ryan (2012).

Previously, it has been suggested that in the early stages of tumour development, cancer cells undergo a higher rate of protein synthesis than protein degradation (Cuervo, 2004; Kondo et al., 2005). Several oncogenes have been

reported to inhibit autophagy especially through mTOR activation (Maiuri et al., 2009), in agreement with the hypothesis that catabolic processes are often suppressed in tumours to support cell mass production. Moreover, autophagy reduced the mutation rate and restrains oncogenesis through the elimination of damaged organelles that produce genotoxic stresses, such as free radicals (Edinger and Thompson, 2003; Kondo et al., 2005). Therefore, defective autophagy during early stages of cancer formation supports tumour growth through increased rate of protein synthesis and genotoxic stresses (Kondo et al., 2005).

The initial evidence that linked tumour suppression to autophagy comes from the deletion of *Beclin 1* in various breast cancer cell lines (Aita et al., 1999). Meanwhile, re-expressing Beclin 1 protein restores autophagy and represses tumourigenesis in human MCF7 breast carcinoma cells (Liang et al., 1999). Notably, *Beclin 1* knockout mice died early during embryogenesis (Yue et al., 2003), while the autophagy defective heterozygous *Beclin 1*-deficient mice develop higher incidence of lymphoma, hepatocellular carcinoma and lung cancer (Qu et al., 2003; Yue et al., 2003). In addition, autophagy defective mice with systemic mosaic deletion of *Atg5* and liver-specific *Atg7* deficient mice develop benign liver adenomas (Takamura et al., 2011). These suggest that autophagy is important in suppressing tumour growth, at least at the early stage of tumour growth (Kondo et al., 2005). Moreover, impaired autophagy regulation in apoptosis-defective tumours stimulates angiogenesis, necrotic cell death and inflammation in promoting tumour growth (Degenhardt et al., 2006).

Mounting evidence has also indicated that autophagy is required for tumour progression, such as seen in pancreatic cancer cells (Yang et al., 2011) and Ras-expressing cell lines (Guo et al., 2011). Despite the suppression of autophagy in the early stage of tumour growth, autophagy seemed to be upregulated in the later stages of tumour development to protect cells (Kondo et al., 2005). During cancer advancement, cells have to survive in limited nutrients and oxygen (hypoxia) conditions as blood supply is reduced due to poor vasculature (Hockel and Vaupel, 2001; Ogier-Denis and Codogno, 2003; Shankar et al., 1996). These results in metabolic stress in cancer cells and subsequently activate autophagy (Shay and Celeste Simon, 2012). This is in line with autophagy's pro-survival role in sustaining cells that are deprived of serum, amino acids or growth factors (Boya et al., 2005; Lum et al., 2005). For example, one early study on human colon cancer cells found that these cells are able to sustain under nutrients scarcity and also have high autophagy levels (Houri et al., 1995). Moreover, specific lines of tumour cells are also found to be unusually tolerant to nutrients deprivation (Izuishi et al., 2000).

Under limited oxygen supply, hypoxia is able to activate autophagy through hypoxia-inducible factor-1 α (HIF1α)-independent effects (DeYoung et al., 2008). The activated HIF1α induces ER stress, and subsequently activated autophagy eliminates excess ER and reduce mitochondrial mass for adaptations to hypoxic conditions (Glick et al., 2010; Janku et al., 2011). Also, autophagy is able to protect cells from anoikis upon detachment from the extracellular matrix, suggesting a possible role of autophagy in metastasis and in supporting tumour

progression (Fung et al., 2008). Moreover, autophagy level is elevated in many solid-tumours (Mathew et al., 2009), highlighting the importance of autophagy as a pro-survival strategy for cells. In support of this hypothesis, the poor outcome of radiation therapy or chemotherapy-resistance of tumour cells is usually linked to elevated level of autophagy. For example, chemotherapy-induced cytotoxicity that activates autophagy releases high-mobility group box 1 (HMGB1), which confers for the resistance of leukemia cell lines to chemotherapy (Liu et al., 2011).

1.1.3.4 Autophagy in immunity

Remarkably, selective autophagy is an essential defensive system against microorganisms via selective delivery of microorganisms to degradative lysosomes (known as xenophagy) or delivery of foreign materials of the microbes such as nucleic acids and antigens to endolysosomal compartments for the activation of innate and adaptive immunity (Levine et al., 2011). The p62 and ubiquitin mechanisms are utilised to selectively remove infections by intracellular bacteria such as *Salmonella typhimurium* (Zheng et al., 2009) and *Listeria monocytogenes* to autophagosomes (Yoshikawa et al., 2009). Autophagy is also utilised during human immunodeficiency virus (HIV) replication (Killian, 2012), implicating a significant therapeutic benefit against microorganisms invasion. The HIV protein Nef interacts with Beclin 1 to inhibit autophagosome maturation, which subsequently protects HIV from degradation (Kyei et al., 2009). Moreover, the ICP34.5, a herpes simplex virus type 1 (HSV-1)-encoded neurovirulence protein, binds to Beclin 1 to inhibit its autophagy function during immune invasion (Orvedahl et al., 2007). The Toll-like receptors (TLR) and nucleotide

oligomerization domain (NOD)-like receptors (NLR) are also involved in the regulation of autophagy process, in addition to their mediation of pro-inflammatory cytokine production (Shi and Kehrl, 2008; Travassos et al., 2010). Moreover, other immune system signals such as T helper 1 (Th1) cytokines interferon (IFN)-γ and tumour necrosis factor-α (TNFα) as well as CD40 signaling stimulates autophagy, while Th2 cytokines such as interleukin (IL)-4 and IL-13 inhibit autophagy (Harris et al., 2007).

Autophagy is also involved in mediating adaptive immunity. For example, it is essential in regulating the survival and differentiation of T-and B-cells as well as in Paneth cell homeostasis (Bell et al., 2008; Cadwell et al., 2008; Miller et al., 2008; Pua et al., 2009). Autophagy also helps to coordinate in the antigen presentation of major histocompatibility complex (MHC) class I and class II during infections by foreign particles (Crotzer and Blum, 2010; English et al., 2009; Lee et al., 2010a). Meanwhile, autophagy facilitates the delivery of cytoplasmic viral nucleic acids to endosomal TLR to stimulate the production of type 1 IFN and IFN-dependent immune responses (Lee et al., 2007c). Furthermore, immunization in cells induced with autophagy enhances the competency of cross-priming in antigen-specific $CD8^+$ T cells (Uhl et al., 2009). In addition, autophagy is also crucial in the modulation of self-tolerance and specific T-cell selection (Nedjic et al., 2008).

1.1.3.5 Autophagy in differentiation and development

Autophagy also plays an essential role in cellular differentiation and development, enabling extensive cellular and tissue remodeling through

degradation process (Mizushima and Levine, 2010). Earlier studies have shown that autophagy is required for spores formation in starved yeasts (Tsukada and Ohsumi, 1993) and dauer formation in *Caenorhabditis elegans* during starvation (Melendez et al., 2003); chick retina development (Mellen et al., 2008), clearance of apoptotic cells in embryonic development (Qu et al., 2007), *Leishmania major* differentiation (Besteiro et al., 2007), proliferation and differentiation in intestinal protozoan parasite (Picazarri et al., 2008), and also recently through GATA-1, the main regulator of hematopoiesis that directly activates gene transcription of LC3 (Kang et al., 2012) as well as in encystations of *Acanthamoeba castellanii* (Song et al., 2012). Moreover, autophagy also plays a major role of differentiation and development in liver, brain, intestines, heart, lung, skeletal muscle, kidney, pancreas, and bone as reviewed recently (Mizushima and Komatsu, 2011).

1.1.3.6 Autophagy and ageing

As autophagy is able to renew cells, therefore its ability to prevent ageing and promote longer lifespan is much correlated. At present, numerous reports have showed the anti-ageing effects by TOR suppression (Lamming et al., 2012; Powers et al., 2006; Tatar et al., 2003; Vellai et al., 2003), calorie restriction, activation of sirtuin (Bordone and Guarente, 2005; Yuan et al., 2012), or reduction of insulin-receptor pathway (Lewis et al., 2010). In agreement that autophagy slows down ageing process, a reduction of formation and elimination of autophagosomes is often seen in ageing cells (Terman, 1995). Therefore, declining autophagy level increases the accumulation of detrimental protein aggregates and impaired organelles, resulting in an increase of reactive oxygen

species (ROS), incremental risks in DNA mutations as well as weakened biological processes that lead to disrupted autophagic processes and finally promote ageing (Cuervo et al., 2005).

It is interesting to learn that autophagy plays numerous roles in the physiological state, including cell death, in which will be discussed shortly. Thus, autophagy can be modulated in response to various conditions seen in diseased states such as starvation, hypoxia or other types of metabolic stress (Wilkinson and Ryan, 2010) and serves as an important target for numerous therapeutic benefits.

1.2 Cell death

Cell death was first observed as early as in 1842 (Vogt, 1842), forgotten but subsequently revived by a review written by Glucksmann in 1951 (Glucksmann, 1951). The "programmed cell death" (PCD) is the most common term used in the study of cell death (Vaux, 2002), and was first coined by Lockshin and Williams (1965) to study developmental cell death in insects. Thereafter, cell death is recognised an important mechanism to remove damaged, unhealthy and unneeded cells to regulate normal physiological developments, tissue homeostasis, and acts as a defense system against immunity, inflammation and tumourigenesis (Fuchs and Steller, 2011; Takeda et al., 2007). There are three types of cell death, which are apoptosis, necrosis and autophagic cell death (Clarke, 1990), which will be discussed in more details below.

1.2.1 Apoptosis

Apoptosis (also known as type I cell death) was first termed by Kerr *et al.*,

(1972) to morphologically define this form of cell death. Apoptosis is characterised by the shrinkage of cell, nuclear condensation and fragmentation, followed by engulfment of nearby phagocytes (Kerr et al., 1972). In addition, the presence of phosphatidylserine residues on the outer plasma membrane (Martin et al., 1995) and activation of caspases (Villa et al., 1997) also serve as the hall marks of apoptosis. Apoptosis occurs either through extrinsic or intrinsic mitochondrial pathway, in which death executing signals involves the activation of cell surface receptors by an extracellular death signal or through death signals that arise within the cell, respectively (D'Amelio et al., 2010; Krammer et al., 2007). The Cysteinyl ASPartate-specific proteASE (caspases) are cysteine proteases synthesised in cells as inactive zymogens that cleave target substrates at specific sites of aspartate residues to inactivate pro-survival proteins and to trigger apoptosis (D'Amelio et al., 2010; Shi, 2004; Venderova and Park, 2012). Therefore, activation of caspase is often used as an apoptosis marker (Venderova and Park, 2012). Caspases are divided into two types; one being the apoptosis-initiator caspases comprising caspase -8 and -9, while the other is the executioner caspases, which are of caspase-3,-6 and -7. Inactive initiator caspase monomers are activated with dimerization formation while executioner caspases exists in inactive dimers. Executioner caspases are activated after cleavage by initiator caspases to form enzymatically active dimers (Tait and Green, 2010). Figure 1.3 describes the regulation of both intrinsic and extrinsic apoptosis.

Extrinsic pathway is triggered by the binding of death ligands to specific trimeric cell surface receptors named as death receptors. For example, tumour

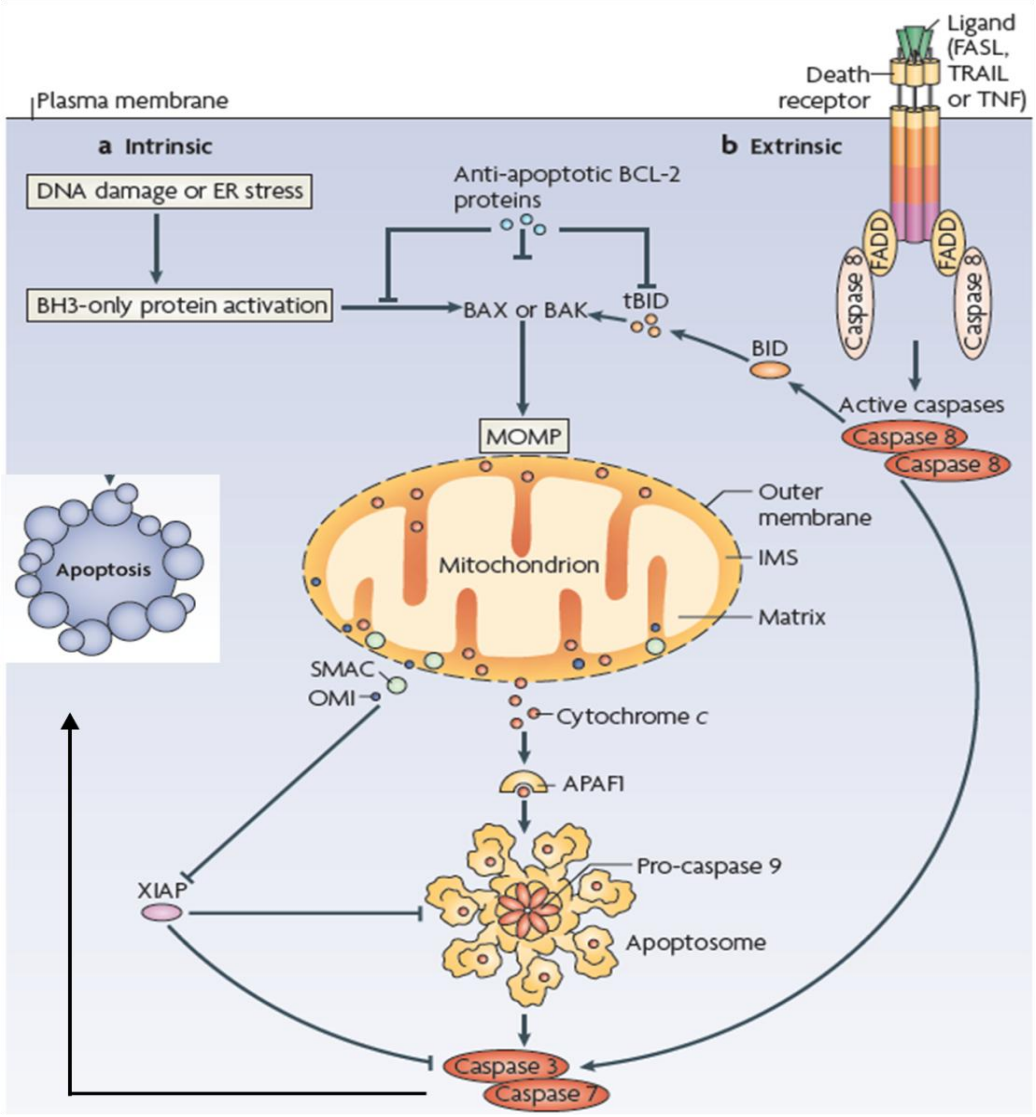


Figure 1.3 Intrinsic and extrinsic apoptosis (Tait and Green, 2010).

necrosis factor (TNF) α (TNFα), TNF (ligand) superfamily member 10 (TNFSF10) or also known as TNF-related apoptosis inducing ligand, TRAIL), FAS/CD95 ligand (FASL/CD95L) bind to respective death receptors such as TNFα receptor 1 (TNFR1), TRAIL receptor (TRAILR) 1 and 2, and FAS/CD95 (Galluzzi et al., 2012). Following receptor trimerization and the clustering of intracellular cytoplasmic death domains, numerous adaptor molecules will then be

recruited to form the death-inducing signaling complex (DISC) (Fulda and Debatin, 2006; Long and Ryan, 2012). In the Fas, TRAILR1 and TRAILR2 pathways, FAS-associated death domain (FADD) and procaspase-8 are associated with the DISC. Meanwhile, the DISC downstream of TNFR1 signaling includes additional molecules comprising TNFR-associated death domain (TRADD) and TNFR-associated factor 2 (TRAF2) (Fulda and Debatin, 2006; Long and Ryan, 2012). Subsequently, the formation of DISC leads to caspase-8 oligomerization and activation followed by death signals that are induced through a series of effector caspases-3, -6 and/or -7 cascade or through mitochondrial outer membrane permeabilization (MOMP) pathway (Long and Ryan, 2012). Upon MOMP, the release of various proteins from the mitochondrial intermembrane space (IMS) subsequently promote caspase activation and apoptosis (Tait and Green, 2010). Meanwhile, the release of second mitochondria-derived activator of caspase (SMAC; also known as DIABLO) and Omi (also known as HTRA2) from the mitochondria neutralizes the caspase inhibitory function of X-linked inhibitor of apoptosis protein (XIAP) (Tait and Green, 2010).

Intracellular stress conditions such as oxidative stress, DNA damage, excessive cytosolic Ca^{2+} and ER stress are able to trigger intrinsic apoptotic pathway (Galluzzi et al., 2012). These stress stimulates MOMP to cause leakage of mitochondrial proteins through i) irreversible loss of mitochondrial transmembrane potential with cessation of ATP production in mitochondria, ii) toxic protein discharge from mitochondria IMS into the cytosol and iii) inhibition of respiratory chain. Intrinsic pathway has been recently reviewed to be

differentiated into two subgroups, which are caspase-dependent and caspase-independent intrinsic apoptosis based on the magnitude of cytoprotection due to caspase inhibition (Galluzzi et al., 2012). Subsequent to stimulated mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* (cyt *c*) released from the mitochondria will promote apoptosis protease-activating factor-1 (APAF-1) and ATP/dATP to assemble the apoptosome that aids the proteolytic maturation of caspase-9 from procaspase-9. Mature caspase-9 remains associated to apoptosome while involved in recruiting and activating executioner caspase-3 and/or caspase-7. Both extrinsic and intrinsic signaling converge at caspase-3 and together with other effector caspases (for example, caspase-7 and caspase-6), to cleave specific substrates and eventually cell death (D'Amelio et al., 2010).

1.2.1.1 Autophagy and apoptosis

At present, the role of autophagy and apoptosis seemed complex, as it is generally known for its pro-survival role, while paradoxically, it also able to mediate apoptosis under certain circumstances. For example, the critical pro-survival role of autophagy has been demonstrated in various studies that utilize Atg-knockout mice including mice deficient of *Atg3*, *Atg5*, *Atg7*, *Atg9* and *Atg16L*. These mice, which are defective in autophagy, subsequently die on the day one of birth due to starvation despite the normal appearance at birth (Komatsu et al., 2005; Kuma et al., 2004; Saitoh et al., 2009; Saitoh et al., 2008; Sou et al., 2008). Moreover, mice of neuron-specific *Atg5* or *Atg7* knockouts suffer from apoptotic neuronal deaths as well as neurodegeneration, while T-cell specific of Atg5 deficiency observes an increase apoptosis in peripheral T-cells during T-cell

activation (Hara et al., 2006; Komatsu et al., 2006; Pua et al., 2009).

Despite the much understood role of autophagy in protecting cell death, the pro-death function of autophagy has also been implicated in several studies. For example, the overexpression of Atg1 lead to high levels of autophagy and caspase-dependent cell death in *Drasophila* (Scott et al., 2007). Moreover, during *Drasophila* development, autophagy contributes to cell death in several different tissues types including salivary gland, midgut, and in reproductive cells (Berry and Baehrecke, 2007; Denton et al., 2009; Hou et al., 2008; Nezis et al., 2010). Also, in a model of HIV infection, HIV-1 envelope glycoproteins-mediated autophagy is required to trigger $CD4^+$ T cell apoptosis in which apoptosis was blocked when autophagy was inhibited (Espert et al., 2006).

The pro-death function of autophagy in apoptosis seemed to be regulated by Atg genes. For example, fragments of typical Atg proteins formed from proteolytic cleavage such as truncated Atg5, Atg4D and Beclin 1 are found to localize at mitochondria to trigger the release of pro-apoptotic factors from mitochondria and subsequently stimulate apoptosis (Betin and Lane, 2009; Wirawan et al., 2010; Yousefi et al., 2006). Moreover, Atg5 binds to FADD to trigger IFN-γ-induced cell death (Pyo et al., 2005) while Atg7 participates in lysosome-dysfunction-induced apoptosis in neural cells (Walls et al., 2010). In addition, Beclin 1 enhances apoptosis in response to obatoclax, a Bcl-2 inhibitor while in the same study; Atg5 downregulation reduces cell death, despite the unaltered appearance of cleaved caspase-3 (Heidari et al., 2010). The conjugation of Atg12-Atg3 could also sensitize cells downstream of mitochondrial signaling

(Radoshevich et al., 2010).

In the crosstalk between apoptosis and autophagy, the interaction of the proteins involved in these two signaling phenomena is crucial in determining the final cell fate. For example, Bcl-2 and B-cell lymphoma-extra large (Bcl-xL), are members of the anti-apoptotic Bcl-2 family that inhibit the autophagic activity by binding to Beclin 1 via the Bcl-2 homology (BH)-3 domain of Beclin 1 (Levine et al., 2008; Liang et al., 1998). However, the binding of Beclin 1 to Bcl-2 does not affect the anti-apoptotic role of Bcl-2 (Ciechomska et al., 2009). Meanwhile, the interaction of Ambra1 with Bcl-2 at the mitochondria is also able to inhibit autophagy by preventing Ambra1 from enhancing Beclin 1 activity (Strappazzon et al., 2011). Also, FLICE-inhibitory protein (FLIP) is able to suppress autophagy via binding to Atg3 and thus attenuate LC3 lipidation (Lee et al., 2009).

In contrast to the earlier studies, pro-apoptotic proteins seem to stimulate autophagy as well. For example, several BH3-only proteins such as Bcl-2-associated death promoter protein (Bad), truncated BH3 interacting domain death agonist (tBid), and BNIP3 compete with Beclin 1 for Bcl-2 binding, and thus abolish the Bcl-2-Beclin 1 interaction (Lamparska-Przybysz et al., 2005; Luo and Rubinsztein, 2010; Mazure and Pouyssegur, 2009). In addition, the phosphorylation by death-associated protein kinase (DAPk) promotes Beclin 1 release from binding to Bcl-xL (Zalckvar et al., 2009) as well as c-Jun N-terminal kinase (JNK) phosphorylation of Bcl-2 to relief the sequestered Beclin 1 from Bcl-2 protein (Wei et al., 2008). It is also interesting to note that caspases, such as caspase-9, promotes autophagic flux possibly by regulating lysosomal pH and

cathepsin activity (Jeong et al., 2011).

Meanwhile, other apoptotic proteins seem to stimulate autophagy as well. For example, several BH3-only proteins such as Bcl-2-associated death promoter protein (Bad), truncated BH3 interacting domain death agonist (tBid), and BNIP3 compete with Beclin 1 for Bcl-2 binding, and thus abolish the Bcl-2-Beclin 1 interaction (Lamparska-Przybysz et al., 2005; Luo and Rubinsztein, 2010; Mazure and Pouyssegur, 2009). In addition, the phosphorylation by death-associated protein kinase (DAPk) promotes Beclin 1 release from binding to Bcl-xL (Zalckvar et al., 2009) as well as c-Jun N-terminal kinase (JNK) phosphorylation of Bcl-2 to relief the sequestered Beclin 1 from Bcl-2 protein (Wei et al., 2008).

Another interesting finding is the involvements of p53 in apoptosis and autophagy, as p53 is a well-known pro-apoptotic tumour suppressor and its contrasting roles in autophagy. The cytoplasmic p53 suppresses autophagy possibly through ER stress signaling, as inactivation of inositol requiring 1α (IRE1α) prevents the activation of autophagy following p53 neutralisation (Tasdemir et al., 2008). Meanwhile, nuclear p53 stimulates autophagy through the transactivation of damage-regulated autophagy modulator (DRAM), a p53 target gene that is activated during DNA damage and is involved in encoding lysosomal protein (Crighton et al., 2006). Moreover, p53 has additional role in inhibiting mTORC1 through AMPK signaling (Feng et al., 2005), which subsequently induces autophagy. On the other hand, the p62 protein is also implicated in the regulation of autophagy and apoptosis. Cullin3 (CUL3), an E3 ligase, initially mediates the polyubiquitination of caspase-8. Subsequently, p62 promotes the

aggregation of CUL3-modified caspase-8 within p62-dependent foci to enhance apoptosis (Jin et al., 2009).

1.2.2 Necrosis

Necrosis (also known as type III cell death) was earlier known as a form of unregulated, accidental cell death that do not resemble the morphological traits of either apoptosis or autophagy, before known lately that it is also a regulated process of death (Galluzzi et al., 2012). Necrosis is also described as an "ordered cellular explosion", which is morphologically characterised by an early onset of plasma membrane permeabilisation, swelling and the final rupture of the cells will causes the release of intracellular structures to the surroundings. In contrast to apoptotic cells, the nuclei of necrotic cells remain intact (Vandenabeele et al., 2010). This process can be stimulated by alkylating DNA damage agents, excitotoxins, ligation of death receptors and caspase inhibition (Cho et al., 2009; Galluzzi et al., 2012; He et al., 2009; Zhang et al., 2009a).

Receptor interacting protein kinase-1 (RIP1) and -3 (RIP3) interactions are known to be critical regulators of necrosis in which RIP3 is recruited to RIP1, thus forming a complex for induction of necrotic cell death (Cho et al., 2009; He et al., 2009; Zhang et al., 2009a). More recently, the understanding of programmed necrosis regulation has improved tremendously since the discovery of necrostatin-1 as a potent inhibitor for necroptosis, a term used specifically for programmed necrosis (Degterev et al., 2005). Figure 1.4 describes the necroptosis signaling. Meanwhile, another form of necrotic cell death that is much being in focus recently is the poly-(ADP-ribose) polymerase (PARP)-mediated cell death.

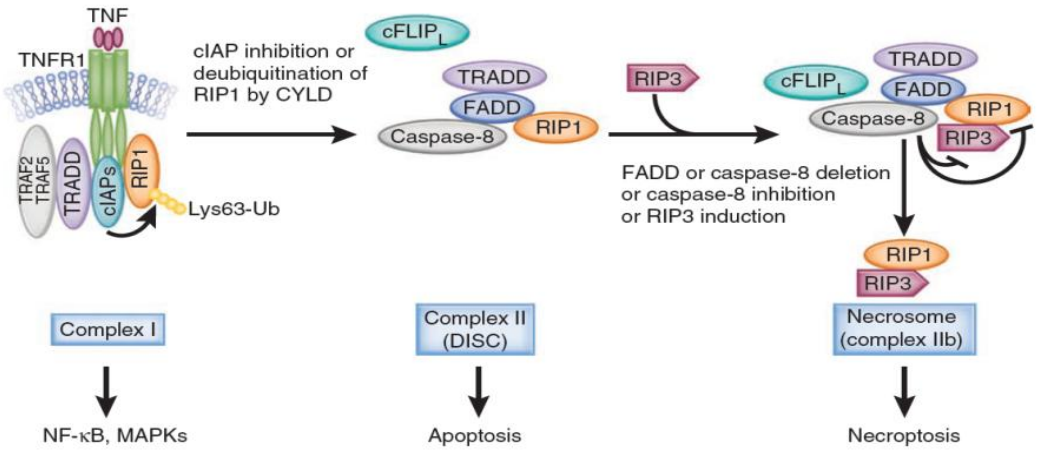


Figure 1.4 The necroptosis signaling (Han et al., 2011).

The TNFR1 is a receptor that regulates cell survival, inflammation, apoptosis and necrosis depending on the subsequent downstream signals coordination. Bound TNF causes TNFR1 to undergo conformational changes that enable the recruitments of numerous signaling molecules such as TRADD, TRAF2, cellular inhibitor of apoptosis 1 (cIAP1), and RIP1 to form a complex termed as complex I, in which RIP1 is heavily ubiquitinated by E3 ligases at lysine63-linked polyubiquitination (Lys63-Ub) by cIAP ligases. This complex formation is responsible for nuclear factor kappa B (NF-κB) activation for cell survival (Chan and Baehrecke, 2012) and mitogen-activated protein kinases (MAPK) (Han et al., 2011). TRAF5 is also involved in interacting with TRAF2 to mediate the downstream NF-κB activation (Aizawa et al., 1997). Deubiquitination of RIP1 by cylindromatosis (CYLD) or inhibition of cIAP proteins promotes the conversion of complex I to complex II and inhibits NF-κB activation (Han et al., 2011; Wright et al., 2007). Uninhibited and active caspase-8 will mediate the cleavage of RIP1 and RIP3 to generate caspase cascade and apoptosis (Lin et al.,

1999). Meanwhile, in the presence of cellular form of the caspase-8 inhibitor FLIP (cFLIP)-long form ($cFLIP_L$), the activity of caspase-8 is inhibited, thus promoting RIP1 and RIP3 interaction to form necrosome (also known as complex Iib) (Han et al., 2011). In the event of caspase-8 inactivation, FADD or caspase-8 depletion, or induction of RIP3 signaling, the inhibition on RIP1-RIP3 is freed to form necrosome and subsequently induces necroptosis in TNF-treated cells (Han et al., 2011).

More recently, the identification of additional complexes involved in the regulation of necroptosis has shed more light into this area. For example, upon necroptosis induction by TNFα and ROS, the mitochondrial protein phosphatase phosphoglycerate mutase 5 (PGAM5) is responsible for activating the GTPase activity of the mitochondrial fission factor dynamin-related protein 1 (Drp1) to cause mitochondrial fragmentation, which is an essential step for necrosis induction (Wang et al., 2012). In addition, an adaptor protein Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF/TICAM-1) forms a complex with RIP3 upon TLR3/TLR4 activation as it is required for TLR3/TLR4-induced necrosis in innate immunity, indicating an important role of programmed necrosis during microbial invasion in mammals (He et al., 2011). Moreover, RIP3 is also found to phosphorylate mixed lineage kinase domain-like (MLKL) protein, in which the phosphorylations are critical for downstream RIP3 regulation in necrosis (Sun et al., 2012).

Meanwhile, the PARP-mediated necrosis is another form of necrotic cell death that is activated during DNA damage, due to DNA strand breaks in

response to oxidative stress/ROS or DNA-damaging agent such as DNA alkylating agents (Zong et al., 2004). PARP consists of a family of nuclear enzymes that regulate chromatin structure, transcription, and gene transcription, which are controlled through poly(ADP-ribosyl)ation (Jagtap and Szabo, 2005; Krishnakumar and Kraus, 2010; Shen and Codogno, 2012). The necrotic cell death is triggered by PARP in the event of DNA damage; causing rapid ATP depletion, translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus and eventually, necrosis (Ha and Snyder, 1999; Yu et al., 2002).

The discovery of necroptosis as an additional or alternate programmed pathway in cell death is particularly significant as it can possibly be linked to tumour suppression; as necrosis is often observed in chemotherapy treatments and apoptosis-deficient tumour cells (Long and Ryan, 2012).

1.2.2.1 Autophagy and necrosis

Similarly to apoptosis, the regulation of autophagy in necrosis seems to be rather complex as well. The pro-survival and pro-death roles of autophagy are also associated with necroptosis. Firstly, autophagy protects apoptosis-deficient cells from necrotic cell death following ischemia (Degenhardt et al., 2006). In the event of autophagy impairment in apoptosis-deficient cells, necrotic cell death ensues and necrotic inflammation and tumour growth are upregulated (Degenhardt et al., 2006). Also, autophagy is able to inhibit necroptosis in various cell lines, such as in L929 cells or human cancer cells that are stimulated with TNFα or starvation (Farkas et al., 2011; Ye et al., 2011).

In contrast, autophagy-dependent necroptosis characteristics are observed

in acute lymphoblastic leukemia cells when the cells are stimulated with a combination of glucocorticoid dexamethasone and rapamycin, indicating that in such system, autophagy promotes necroptosis (Bonapace et al., 2010). Moreover, in *C. elegans*, the inactivation of *Atg* genes seems to reduce necrotic cell death (Samara et al., 2008). Meanwhile, the role of pan-caspase inhibitors such as zVAD-fmk (zVAD) in inducing necroptosis or autophagic cell death in L929 cells has been controversial (Wu et al., 2009; Yu et al., 2004). In fact, zVAD is found to trigger necroptosis in the L929 cells, which are deficient in apoptotic machinery due to the suppression of autophagy mediated by zVAD (Wu et al., 2008).

On the other hand, in the PARP-mediated necrosis, a number of studies have indicated that PARP-activation is coupled with autophagy to regulate a pro-survival role during DNA damage induction (Albert et al., 2007; Huang and Shen, 2009; Hwang et al., 2010; Munoz-Gamez et al., 2009). The possible mechanism in which PARP activates autophagy lies on the AMPK activation under ATP depletion conditions, as AMPK is readily activated when ATP levels are low (Hardie, 2007). AMPK plays a vital role in autophagy, as AMPK phosphorylates ULK1 directly to initiate autophagy (Egan et al., 2011; Kim et al., 2011; Lee et al., 2010c; Shang et al., 2011). Meanwhile, PARP also mediates the AIF nuclear translocation during ATP depletion, and therefore the dual-role of PARP in deciding the final cell fate lies on the forces that balances the outcome, depending on the extent of the DNA damage and the functional role of PARP (Shen and Codogno, 2012).

Despite the contradicting roles of autophagy in necrotic-mediated cell death, the molecular link between autophagy and necrosis remains to be further investigated.

1.2.3 Autophagic cell death

The term "autophagic cell death", also known as type II cell death, has been used widely to morphologically define instant cell death that is accompanied by enormous cytoplasmic vacuolization along with increased autophagic flux (Kroemer et al., 2009). Recently, this type of cell death is redefined as cell death that can be suppressed when autophagic pathways are pharmacologically or genetically inhibited, increased autophagic flux or an increased degradation of autophagic substrates such as p62 (Galluzzi et al., 2012). In fact, the autophagic cell death term should be used when the cell death is caused by autophagy and not cell death with autophagy, as autophagy may be present as an attempt for the cells to prolong survival (Shen and Codogno, 2011). In line with this, Shen and Codogno (2011) have proposed that the following criteria should be met for the definition of cell death by autophagy: i) cell death that occur is independent of apoptosis, ii) observation of increased autophagic flux instead of autophagic markers in the dying cells and, iii) suppression of autophagy enhances cell survival (Shen and Codogno, 2011).

Autophagic cell death has been observed in *Drosophila* salivary glands (Berry and Baehrecke, 2007), and recent studies have showed the involvement of *trol*, a novel gene *CG11880*, and the cop9 signalsome component cop9 signalsome 6, are required for *Drosophila* larval salivary gland degradation

although neither of these are required for autophagy (McPhee et al., 2012). Autophagy has also been showed to be required for the removal of *Drosophila* larval midgut (Denton et al., 2010; Denton et al., 2009; Lee et al., 2002). Although the midgut PCD requires autophagy for removal, however the loss of autophagy seems to delay PCD but does not abolish it. Therefore, much still need to be investigated about the role of autophagy in metamorphosis (Denton et al., 2010).

Moreover, the contrasting roles of Ras expression in promoting cell growth with increasing autophagy (Guo et al., 2011) or in causing autophagic cell death (Elgendy et al., 2011) has further showed the complexity of autophagy in regulating cell death and cell survival. In amoeba *Dictyostelium discoideum*, a distinct role of autophagy is seen under starvation and cell death conditions in which Atg1 is required for the pro-survival role during starvation, but is also required for autophagic cell death during exposure to differentiation factor (DIF-1) under starvation conditions (Luciani et al., 2011). Meanwhile, the development of vascular system through secondary cell wall thickening and cell death of the xylem cells to form the tracheary elements in *Arabidopsis* requires autophagic cell death (Kwon et al., 2010). At present, the more supporting evidence of autophagic cell death comes from the studies of hippocampal neural (HCN) stem cells, following insulin withdrawal treatment in which *Atg7* knockdown blocks cell death, while it is also independent of caspase activation (Yu et al., 2008).

Nevertheless, cell death by autophagy is still relatively uncommon at present (Denton et al., 2009). Despite the pro-death role of autophagy observed

under various cellular settings, autophagy seems to be essential for various functional roles in cellular processes when elicited, instead of contributing negatively in the cellular regulatory functions. However, it is also important to distinguish carefully among the numerous literature cited in the literature, whether autophagy is the cause of cell death or precede for the cells to survive longer. As autophagy is essentially regulated by the mTOR signaling, the mTOR pathway will be discussed next.

1.3 mTOR pathway

1.3.1 Overview of the TSC1-TSC2 and mTOR signaling pathway

The TOR is a serine/threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKK) family which enable organisms to mediate genetic, metabolic, and environmental stresses (Zoncu et al., 2011). The breakthrough of TOR as the target of the immunosuppressant rapamycin, a lipophilic macrolide found in soil bacterium, *Streptomyces hygroscopicus* in Easter Island (Vezina et al., 1975) has led to a substantial amount of studies targeting this pathway subsequently. Figure 1.5 describes the main components in the mTOR signaling.

The mTOR functions as the catalytic subunit that binds to different accessory proteins to form multiprotein mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) respectively (Chi, 2012). mTORC1 comprises of six while mTORC2 has seven distinguished protein complexes respectively (Laplante and Sabatini, 2012).

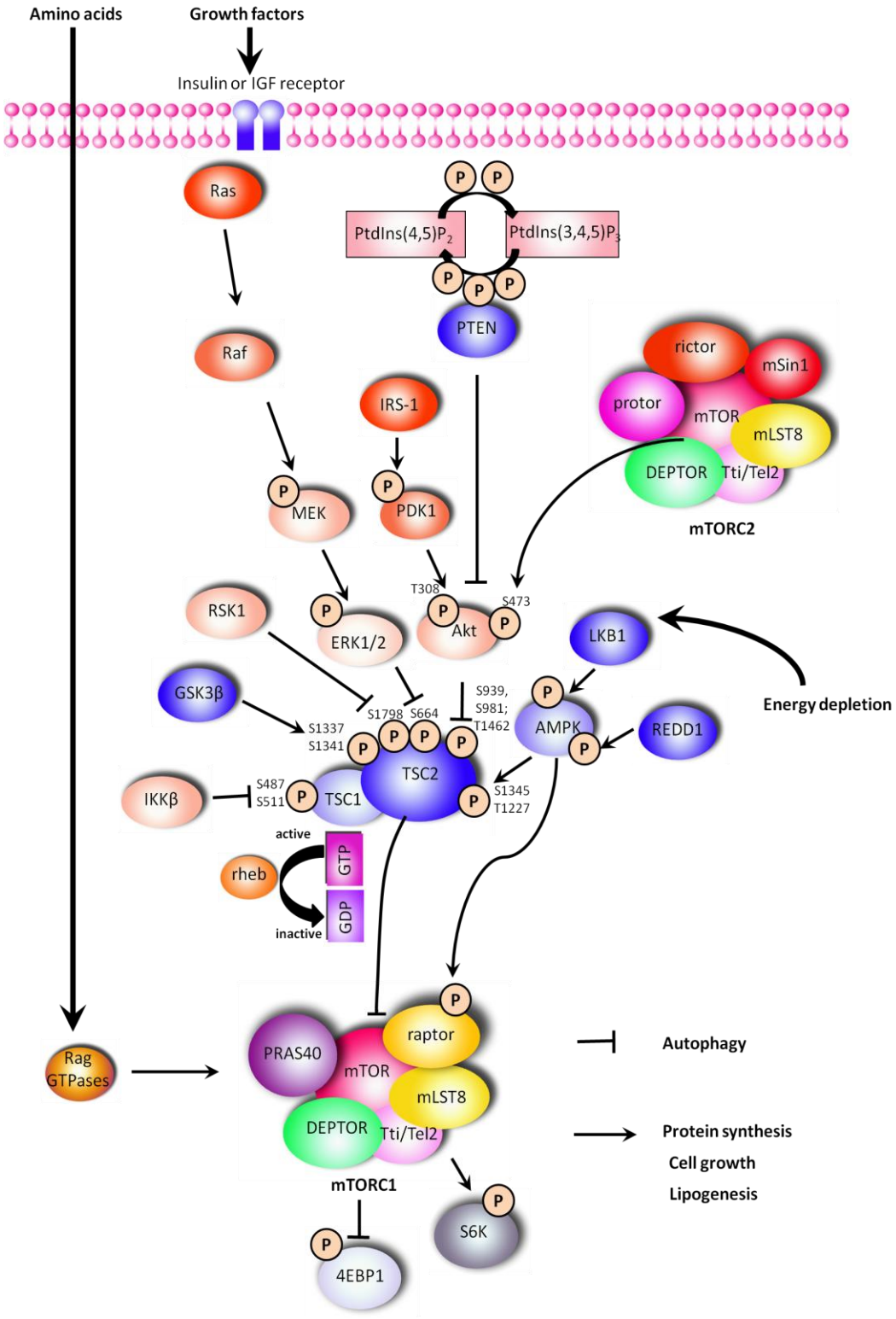


Figure 1.5 The mTOR signaling.

Both complexes have the catalytic mTOR, mammalian lethal with sec-13 protein 8 (mLST8, or GβL) (Jacinto et al., 2004; Kim et al., 2003), DEP domain containing mTOR-interacting protein (DEPTOR) (Peterson et al., 2009), and Tti/Tel2 complex (Kaizuka et al., 2010). Meanwhile, mTORC1 has a scaffolding protein of regulatory associated protein of mTOR (raptor) which is sensitive to rapamycin (Hara et al., 2002; Kim et al., 2002) and proline-rich Akt substrate of 40 kD (PRAS40) (Sancak et al., 2007; Thedieck et al., 2007; Vander Haar et al., 2007; Wang et al., 2007b), while mTORC2 has a different scaffold protein that is insensitive to rapamycin, unless under prolonged treatment, which is known as rapamycin-insensitive companion of mTOR (rictor) (Jacinto et al., 2004; Sarbassov et al., 2004), mammalian stress-activated map kinase-interacting protein 1 (mSin1) (Frias et al., 2006; Jacinto et al., 2006) and protein observed with rictor 1 and 2 (protor 1/2) (Pearce et al., 2007; Thedieck et al., 2007; Woo et al., 2007).

The much more studied mTORC1 is a major regulator for at least five types of cellular signals including amino acids, growth factors, energy, stress and oxygen that in turn, controls protein synthesis, lipogenesis, energy metabolism, autophagy as well as lysosome biogenesis (Laplante and Sabatini, 2012). The TSC1-TSC2 (tuberous sclerosis complex-1, and -2; which are also known as hamartin and tuberin, respectively) forms a functional protein complex that negatively regulates mTOR through the GTPase Activating Protein (GAP) domain in TSC2 by converting active GTP-bound Rheb to inactive GDP-bound Rheb (Inoki et al., 2003a; Tee et al., 2003; Zhang et al., 2003b). Numerous signals

converge at TSC1-TSC2 complex to regulate mTORC1 activity. Growth factors such as insulin and insulin-like growth factor 1 (IGF-1) activates PI3K and Ras pathways. Activation of mTORC1 occurs through the inactivation and phosphorylation of TSC1-TSC2 complex by effector kinases such as protein kinase B (PKB/Akt) (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002) via a negative feedback loop of insulin receptor substrate-1 (IRS-1) proteins (Harrington et al., 2004), extracellular-signal- regulated kinase 1/2 (ERK1/2) (Ma et al., 2005) and ribosomal S6 kinase 1 (RSK1) (Roux et al., 2004).

In contrast, mTORC1 activity is inhibited with the phosphorylation of TSC1-TSC2 complex under low energy conditions and hypoxia by AMPK, which occurs when there is an increase in AMP/ATP ratio (Inoki et al., 2003b). Hypoxia is also able to induce the transcriptional regulation of DNA damage response 1 (REDD1) to activate TSC2 and downregulate mTORC1 (DeYoung et al., 2008; Liu et al., 2009; Zou et al., 2011). Others, such as DNA damage inducers are able to stimulate the *TSC2* and *phosphatase and tensin homolog deleted on chromosome 10* (*PTEN*) expressions (Feng et al., 2005), in which PTEN deactivates Akt while activating AMPK through *Sestrin1/2* thus leading to mTORC1 inhibition through p53-dependent transcription (Budanov and Karin, 2008). TNFα is also able to activate IκB kinase β (IKKβ) that suppreses TSC1 through phosphorylation, leading to mTORC1 activation (Lee et al., 2007b). mTORC1 is also activated by another major regulator of cell growth, proliferation, differentiation, cell polarity and development through the canonical Wnt pathway. Activated Wnt inactivates glycogen synthase kinase 3β (GSK3β)

that usually phosphorylates and promotes TSC2 activity under normal conditions (Inoki et al., 2006). There are also inputs on mTORC1 directly which are independent of TSC1-TSC2 regulation. For example, Akt deactivating phosphorylation on PRAS40, which binds to raptor to inhibit mTORC1 activity through insulin stimulation (Sancak et al., 2007; Thedieck et al., 2007; Vander Haar et al., 2007) and AMPK inhibition of mTORC1 during energy stress through direct phosphorylation on raptor to induce its binding to 14-3-3 (Gwinn et al., 2008).

Amino acid signaling activates mTORC1 activity independent of TSC1-TSC2 (Smith et al., 2005), by utilizing Ras-related GTPases (Rag) (Kim et al., 2008a; Sancak et al., 2008). There are four Rag proteins in mammals, comprising Rag A or Rag B that forms heterodimers with Rag C or Rag D. When Rag A/B is bound to GTP, the complementary Rag C/D bound will be loaded with GDP. Amino acids promote the Rag A/B with GTP through an unknown mechanism, but allow the heterodimer to interact with raptor (Sancak et al., 2008), thus resulting in mTORC1 recruitment to the lysosomal surface through Ragulator. This multicomplex subunit Ragulator scaffolds Rag GTPases to the lysosomal surface and is essential for mTORC1 activation by amino acids (Sancak et al., 2010).

1.3.2 mTOR signaling components and functions

1.3.2.1 mTORC1 function in protein synthesis

The activation of mTORC1 results in promotion of its downstream substrates S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E)-

binding protein 1 (4EBP1) that subsequently control protein synthesis (Ma and Blenis, 2009). The role of mTORC1 in protein synthesis is extensive, such as its vital role in regulating the three classes of nuclear RNA polymerases for ribosome biogenesis (Mayer and Grummt, 2006).The mTORC1-dependent phosphorylation at threonine 389 (T389) of S6K activates its kinase activity in promoting the downstream substrates that are involved in mRNA maturation and protein translation.

In contrast, multisite phosphorylation of 4EBP1 by mTORC1 removes its binding to the translation initiation factor eIF4E that subsequently activates cap-dependent translation (Ma and Blenis, 2009). The 4EBP1 phosphorylation releases eIF4E to form eIF4F complex that is required for cap-binding initiation process. An increase of mRNA biogenesis, translational initiation and elongation ensue following the activation of S6K1 (Ma and Blenis, 2009). In addition, the mTORC1 also stimulates protein synthesis machinery via the activation of transcriptional intermediary factor 1 Alpha (TIF-1A) and thus promoting its interaction with RNA Polymerase I (Pol I) to form transcription-initiation complex (Mayer et al., 2004). In addition, mTORC1 phosphorylates and inhibits Maf1, a Pol III repressor to induce transfer RNA transcription (Kantidakis et al., 2010; Shor et al., 2010).

1.3.2.2 mTORC1 in autophagy and lysosomal regulation

An additional essential role of mTORC1 lies on its regulation on autophagy. In mammals, mTORC1 suppresses autophagy through phosphorylation of ULK1/Atg13/FIP200 complex to initiate autophagy (Ganley et

al., 2009; Hosokawa et al., 2009; Jung et al., 2009), a process that has been discussed in more details earlier in this chapter (Section 1.1.2.1). Another substrate downstream of mTORC1 that is responsible in autophagy regulation includes WD repeat domain phosphoinositide-interacting protein 2 (WIPI2), a mammalian ortholog of yeast Atg18. WIPI2 is a PI3P-mTORC1 effector that positively regulates autophagosomal formation (Polson et al., 2010). Meanwhile, activation of another mTORC1 substrate, death-associated protein-1 (DAP1) has been identified to be a suppressor of autophagy (Koren et al., 2010). The mTORC1 signaling is also found to regulate lysosomes by negatively regulate the transcription factor EB (TFEB), as TFEB controls numerous genes involved in the autophagosome formation to fusion processes, as well as in lysosomal function (Settembre et al., 2011).

1.3.2.3 mTORC1 in lipid regulation

The regulation of lipid is also controlled by mTORC1, implying the various regulatory roles mediated by mTORC1 in the regulation of cell processes. Inactivation of mTORC1 activity causes a reduction in sterol regulatory element binding proteins (SREBP) -1 and -2 expressions which significantly reduce lipogenic genes expressions (Porstmann et al., 2008; Wang et al., 2011) implicating that mTORC1 plays an essential role in lipogenesis. Studies have shown that mTORC1 regulation of SREBPs through S6K (Duvel et al., 2010; Li et al., 2011) and lipin 1 localization (Peterson et al., 2011). The mTORC1 also positively regulate the peroxisome proliferator-activated receptor γ (PPAR-γ) expression which is essential in adipogenesis (Kim and Chen, 2004; Zhang et al.,

2009b).

1.3.2.4 mTORC1 in cellular energy metabolism

mTORC1 positively regulates glycolytic processes through increasing the expression of HIF1, a transcriptional factor (Brugarolas et al., 2003; Duvel et al., 2010; Hudson et al., 2002; Laughner et al., 2001). HIF1 was initially identified through its adaptive role to hypoxia that regulates expressions of anaerobic glycolysis to increase cytosol ATP generation, from the conversion of glucose to pyruvate (Semenza et al., 1994). In addition, it was reported earlier that mTOR itself is an ATP sensor (Dennis et al., 2001). In addition, mTORC1 controls mitochondria biogenesis and oxidative function by regulating the nuclear association between PPAR-γ coactivator 1α (PGC1α) and the transcription factor Ying-Yang 1 (YY1) (Cunningham et al., 2007), although there were recent studies that argued on this study and thus needs further supporting evidences (Laplante and Sabatini, 2012).

1.3.2.5 Functions of mTORC2

Much limited studies have been performed in mTORC2 as compared to mTORC1. mTORC2 was initially discovered to be rapamycin-insensitive that controls cell survival/metabolism and cytoskeleton (Jacinto et al., 2004; Sarbassov et al., 2004) but later, one of the same pioneering groups and another study found that prolonged treatment of rapamycin does inhibit mTORC2 (Phung et al., 2006; Sarbassov et al., 2006). mTORC2 has also been implicated in promoting protein folding and stability of Akt and conventional protein kinase C (PKC) through phosphorylation at the turn motif (TM) I site (Facchinetti et al.,

2008; Ikenoue et al., 2008) as well as its role in activating the serum- and glucocorticoid-induced protein kinase 1 (SGK1) involved in ion transport and growth. mTORC2 also plays a role in terminal differentiation in myoblasts though rictor (Shu and Houghton, 2009). A recent study has linked mTORC2 to ribosomes as binding of ribosomes-mTORC2 increases after insulin activation (Zinzalla et al., 2011).

1.3.3 TSC impairment and implications in pathological diseases

mTORC1 is hyperactivated in the event that TSC1 or TSC2 protein is loss, which is due to the mutation in either of its gene, *TSC1* or *TSC2* respectively. Consequently, tumour formation is promoted in TSC disease. TSC, which is also known as Bourneville disease, was initially described by a French neurologist, Désiré-Magloire Bourneville in year 1880 (Bourneville, 1880). This autosominal dominant disease is a genetic disorder characterised by the formation of hamartomas in numerous organs, including brain (subependymal giant cell astrocytomas [SEGA] and tubers), kidney (angiomyolipomas and cysts), lung (lymphangioleiomyomatosis [LAM]), skin (facial angiofibromas), heart (rhabdomyomas), and retina due to the mutation found in the tumour suppressor genes, *TSC1* or *TSC2* (Gomez, 1991; van Slegtenhorst et al., 1997).

In an older report, TSC disease has an estimated prevalence of 1 in 5800 people (Osborne et al., 1991) and a global prevalence reaching 1 million individuals (Budde and Gaedeke, 2012). Sporadic mutations of TSC genes takes place in about 70-80% cases (De Vries and Bolton, 2000), with phenotypes of *TSC2* mutation being more common and severe comparing to *TSC1* (Sancak et al.,

2005). An implication of two-hit mechanism is present as numerous lesions in TSC patients displayed the loss of both alleles of either *TSC1* or *TSC2* (Au et al., 1999; Green et al., 1994; Henske et al., 1997; Verhoef et al., 1999), although there are also cases of haploinsufficiency for TSC disease pathogenesis (Henske et al., 1996; Niida et al., 2001).

The manifestations of TSC disease were recorded as early as 1900. Benign tumours caused by TSC such as lesions in brains are associated with epilepsy, cognitive retardation, facial angiofibroma, autism, hydrocephalus, and seizures. Other appearances involving kidneys, eyes, heart and lungs that were present were then subsequently reported (Budde and Gaedeke, 2012). There is currently no approved systemic therapies to treat the abnormal biochemistry in TSC disease, such as targeting the upregulated mTORC1 activity (Budde and Gaedeke, 2012), despite the numerous clinical implications of TSC disease, possibly due to the perception that this is a rare disease. However, there are current clinical trials in phase II utilizing sirolimus, an mTORC1 inhibitor, to treat patient with TSC- or LAM-associated angiomyolipomas as well as everolimus to treat SEGAs, with promising results (Budde and Gaedeke, 2012).

1.3.4 Implications of mTOR dysregulation

mTORC1 hyperactivation also leads to numerous other complications. Figure 1.6 describes various diseases, including cancer, that are associated with mTORC1 signaling, implying that this signaling plays highly important roles in the pathological functions of cellular processes.

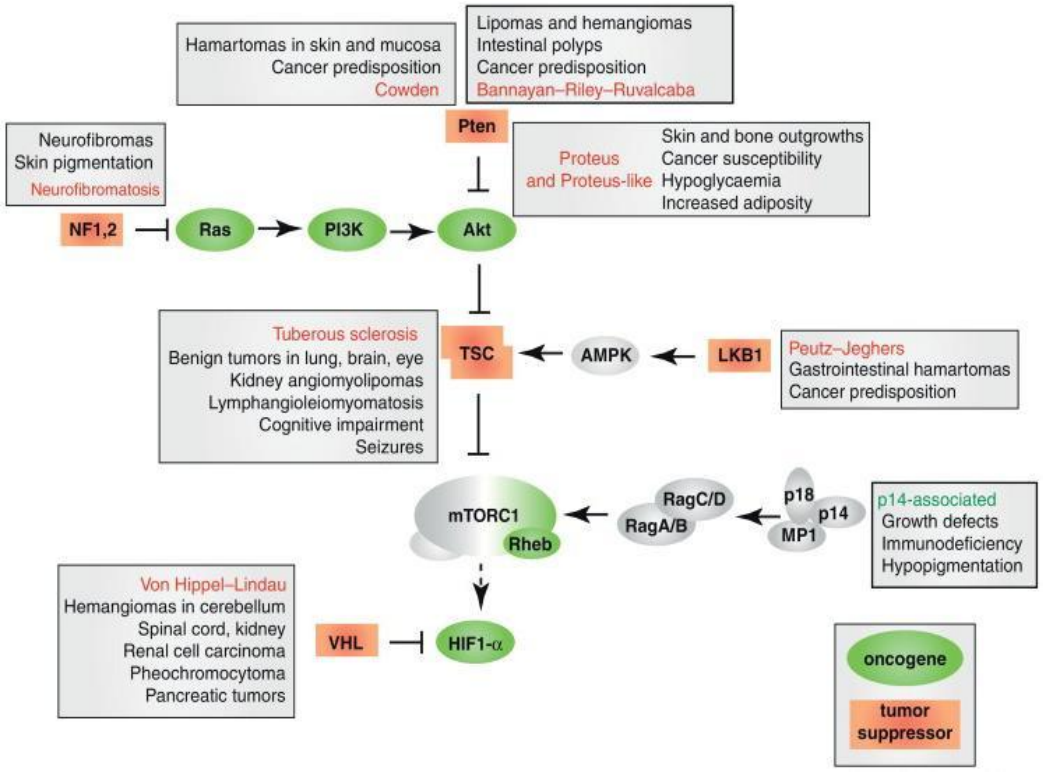


Figure 1.6 mTORC1 signaling and diseases (Efeyan et al., 2012).

Several known tumour suppressor genes such as *p53*, *PTEN*, *LKB1*, *TSC1* and *TSC2* inhibit mTOR activation. In contrast, mTOR activating upstream signaling such as class I PI3K/Akt and ERK1/2 block autophagy and are often hyperactivated in cancers. Thus, autophagy defects may contribute to tumourigenesis (Levine, 2007).

1.4 Oxidative stress and MAPK signaling pathways

1.4.1 Oxidative stress

Oxidative stress refers to the outcome of an imbalance state due to higher accumulated ROS with insufficient antioxidant defenses (Halliwell, 1993). ROS is a group of chemical species produced when incomplete reduction of oxygen takes place, in which it includes superoxide anion (O^{2-}), hydrogen peroxide

(H_2O_2) and the hydroxyl radical (HO•) (D'Autreaux and Toledano, 2007). These species are generated as byproducts from normal cellular processes and atypical metabolic processes in all aerobic organisms. Accumulation of these free radicals can lead to disruption of cellular functions and integrity, such as glutathione depletion, lipid peroxidation, membrane damage and DNA strand breaks (Halliwell, 1993). Cancer-prone inflammatory diseases patients of ulcerative colitis, haemochromatosis and viral hepatitis are linked to free-radical stress (Hussain et al., 2003). Also, there is a frequent pro-oxidative shift in systematic thiol/disulfide redox state in cancer cells, which are termed as "mitochondrial oxidative stress" or "inflammatory oxidative condition" (Singh et al., 2007).

A major source of ROS in the cells is generated at the mitochondrial electron transport chain (ETC) (Chance et al., 1979; Loschen et al., 1971), mainly found through the leakage of electrons that passes the ETC to molecular oxygen (O_2) to form superoxide molecules. These superoxides are then rapidly converted to H_2O_2 spontaneously or by the mitochondrial superoxide dismutase (Mn-SOD) (Alvarez et al., 1987; Boveris and Cadenas, 1975; Loschen et al., 1974). Environmental stimuli such as growth factors, ionizing radiation, ultraviolet (UV) radiation, inflammatory cytokines, oxidizing chemicals, chemotherapeutics, hyperoxia, toxins and transition metals are also able to produce ROS as well as cytosolic enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases that produces superoxides from oxygen (Cui et al., 2012). ROS has been implicated in activating MAPK signaling in numerous studies (McCubrey et al., 2006).

1.4.2 MAPK signaling pathway

The MAPK is a major signaling controlling embryogenesis, differentiation, cell proliferation, death and migration (Kyriakis and Avruch, 2001). MAPK are driven by phosphorylation of three-tier cascades, composing of a i) MAPK, ii) MAPK kinase (MAP2K, MAPKK, MKK or MEK) and a iii) MAPKK kinase or MEK kinase (MAP3K, MAPKKK or MEKK). The stimulation of a MAPK requires the activation of two protein kinases from upstream signaling (Chang and Karin, 2001; Pearson et al., 2001). These comprised of dual specificity, evolutionary conserved enzymes that are able to phosphorylate hydroxyl side chains of serine/threonine and tyrosine residues at Thr-X-Tyr motif that is catalysed by MKKs (Pearson et al., 2001; Wagner and Nebreda, 2009).

The well-characterised mammalian MAPK family is comprised of JNK, p38 MAPK (p38), and ERK1/2. These members are regulated differently by upstream MAPK cascades (Rodriguez-Berriguete et al., 2012). The mammalian MAPK pathways can be activated by various extracellular stimuli through diverse receptor families. These include cytokines or tyrosine kinase receptors that are activated by hormones and growth factors, respectively; vasoactive peptides that stimulate G protein-coupled receptors (GPCR), transforming growth factor beta (TGF)-β-related polypeptides that act through serine/threonine kinase receptors, inflammatory cytokines of the TNF family, as well as environmental stress such as osmotic shock, ionizing radiation and ischemic injury (Kyriakis and Avruch, 2001).

Depending on cell types, the MAPK cascade may be involved in different

cellular response. The MAPK in turn, is deactivated by a variety of MAPK phosphatases (MKPs) with different substrate selectivity (Keyse, 2000; Patterson et al., 2009). Thus, a sustained MAPK activation could involve either the activation by an upstream kinase or inhibition by MKPs. The MKPs are divided based on the subcellular localization, in which the MKPs in the first group are encoded by early genes and localised in nucleus; while the second group is encoded by late genes and could reside in the cytoplasm or both cytoplasm and nuclear compartments (Liu et al., 2007). The JNK and p38 signaling are activated by pro-inflammatory cytokines such as TNFα, IL-6 and IL-1 or anti-inflammatory epidermal growth factor (EGF) and TGF-β cytokines, as well as other cellular stresses comprising genotoxic, osmotic, hypoxic, and oxidative stress (Rodriguez-Berriguete et al., 2012). JNK is activated by upstream MAPKK of MKK4 (also known as JNKK1, SEK1or MEK4) (Derijard et al., 1995; Sanchez et al., 1994) and MKK7 (JNKK2, SEK2 or MEK7) (Tournier et al., 1999; Yao et al., 1997).

The higher MAPKKK tier consists of apoptosis signal-regulating kinases-1 and 2 (ASK1 or MAPKKK5; ASK2 or MAPKKK6) (Nishitoh et al., 1998; Wang et al., 1998), MAPKKK1 (also known as MEKK1) (Xu and Cobb, 1997; Xu et al., 1996), mixed-lineage kinases (MLK) (Kyriakis and Avruch, 2001) and TGF-β-activated kinase 1 (TAK1) (Moriguchi et al., 1996; Ninomiya-Tsuji et al., 1999). Rac and Cdc42, both are small Rho-GTPases, activate the JNK and p38 MAPK signaling but not the ERK pathway (Coso et al., 1995; Khosravi-Far et al., 1995; Minden et al., 1995; Olson et al., 1995). Different MAPKKs such as MKK3 and MKK6 activate p38, but the upstream may be activated by the same

MAPKKKs (such as ASK1 and TAK1) as in JNK signaling (Rodriguez-Berriguete et al., 2012). Meanwhile, ERK1 or ERK2 (ERK1/2) is activated by MKK1/MKK2, in which the upstream involves a Raf isoform such as A-Raf, B-Raf, or Raf-1 (also known as C-Raf) as well as TRAF-2 and TRAF-6 (Rodriguez-Berriguete et al., 2012. The small Ras-like GTPase, Pak activates the Raf-1 kinase; and Ras is activated by the receptor tyrosine kinase (RTK) signaling axis (King et al., 1998; Marshall, 1995).

Activated MAPK signaling then regulates gene expression through a number of mechanisms. For example, MAPK promotes gene transcription histone acetylation to modulate the chromatin structure (Cheung et al., 2000). MAPKs also enhance numerous transcription factors, such as activator protein 1 (AP-1) (Whitmarsh and Davis, 1996), cAMP response element-binding protein (CREB) (Arthur and Cohen, 2000; Arthur et al., 2004; Eliopoulos et al., 2002; Wong et al., 2004), serum response factor (SRF) (Posern and Treisman, 2006), and CCAAT/enhancer binding protein beta (C/EBPβ or also known as NF-IL6) (Nakajima et al., 1993). MAPKs are also involved in regulating gene expression by altering the transport, stability, and translation of mRNAs with AU-rich element (ARE), AUUUA (Carballo et al., 1998; Mahtani et al., 2001; Stoecklin et al., 2004). Moreover, MAPKs are also able to modulate microRNAs (miR) expressions, such as miR-21 (Huang et al., 2009b), miR-125a (Monk et al., 2010), and miR-155 (O'Connell et al., 2007; Yin et al., 2008).

In addition, MAPKs are also capable in regulating the stability of proteins such as the the acetylation of a key haemopoietic transcription factor, GATA-1

for degradation (Hernandez-Hernandez et al., 2006). The downstream signaling subsequently activates a range of cellular responses for growth, survival, cell differentiation and cell death processes. Figure 1.7 shows a simple representative of the MAPK signaling.

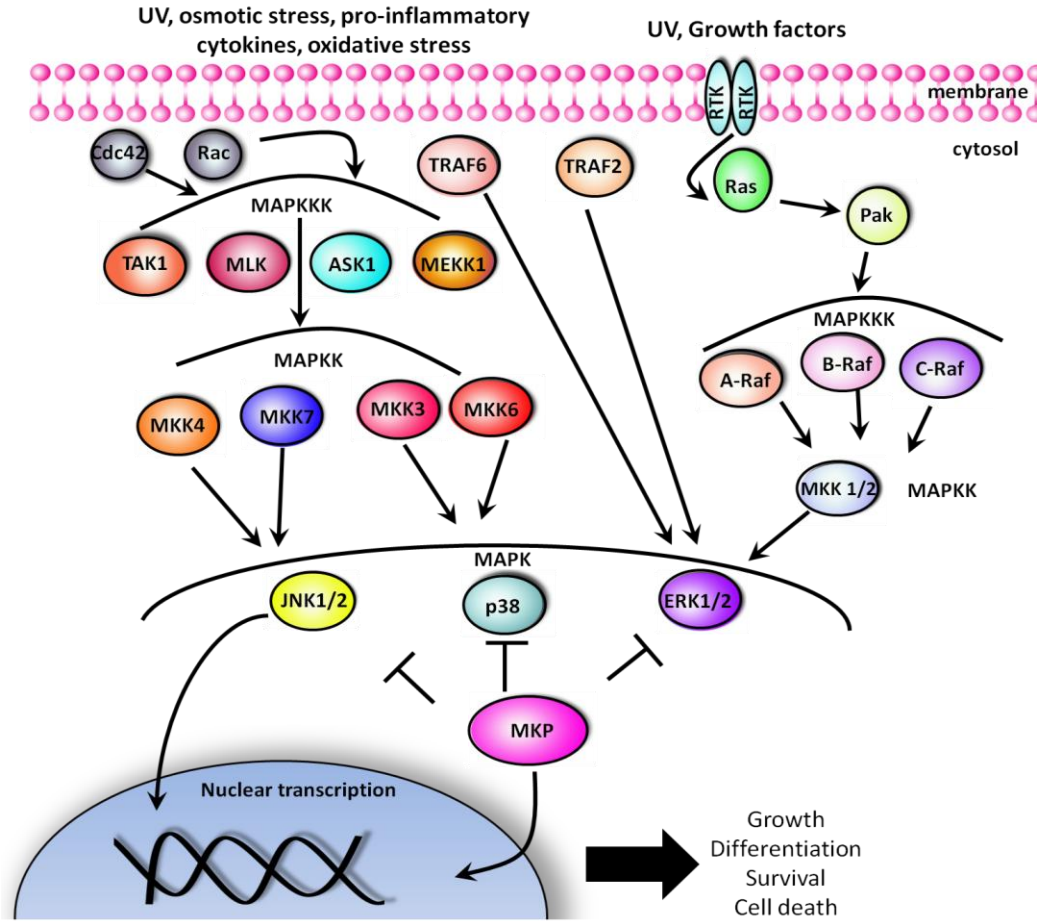


Figure 1.7 The MAPK signaling cascades.

1.4.3 Crosstalk between MAPK and mTOR

Meanwhile, much study on the crosstalk of these two distinct signaling pathways of mTORC1 and MAPK have linked the ERK downstream signaling to the mTORC1 activity. Ras activates Raf to promote the activity of MKK1/MKK2, and subsequently the phosphorylation of MKK1/2 activates ERK1/2 to stimulate

phosphorylation of multiple cytoplasmic substrates such as RSK and nuclear substrates such as transcription factors (Roux and Blenis, 2004). Meanwhile, different isoforms of S6K and RSK are also formed when Akt is activated, through phosphorylation of phosphatidylinositol-dependent kinase 1 (PDK1) that activates Akt at T308 (Alessi et al., 1996). An earlier study that linked MAPK to mTORC1 pathway is first observed in *S6K* deficient mice, as phosphorylated S6 ribosomal proteins are present despite the S6K deficiency in the system, thus linking the existence of an S6K-independent pathway (Pende et al., 2004).

Meanwhile, RSK is also found to be the upstream kinase that activates the eukaryotic translation initiation factor 4B (eIF4B) to mediate its activity in translation initiation process (Shahbazian et al., 2006). Consistently with earlier observations, subsequent study have found that in T cells, the complete blockage of S6 protein phosphorylation requires both mTORC1 and ERK signaling and RSK is required in an S6K-independent manner to mediate S6 phosphorylation (Salmond et al., 2009). mTORC1 is also positively mediated through the raptor phosphorylation by RSK and ERK signaling (Carriere et al., 2008; Carriere et al., 2011).

On the other hand, the IRS-1 is also negatively regulated by JNK in which JNK acts as a negative feedback regulator for insulin action by phosphorylating at S307 in IRS-1 (Aguirre et al., 2000; Hilder et al., 2003; Lee et al., 2003). IRS-1 is also the upstream regulator of PI3K-Akt-mTORC1 signaling, which is regulated by TSC1/TSC2 and mTORC1 through the repression of S6K activity on IRS-1 (Harrington et al., 2004). Cells that are deficient of TSC complex seem to have

lower Akt activation due to the suppression of S6K activity towards IRS-1 (Harrington et al., 2004). Therefore, the convergence of JNK to PI3K-Akt-mTORC1 signaling exists through the IRS-1 regulation. Moreover, more studies have shown that inhibition of insulin and Akt mediated by JNK reduces cell survival, increases insulin resistance, promotes obesity as well as diabetes in a few models (Gao et al., 2003; Hirosumi et al., 2002; Mamay et al., 2003). Nevertheless, the link of TSC1/2-mTORC1 pathway to JNK seems to be elusive at present.

1.4.4 Roles of JNK-mediated ROS signaling in cell death

1.4.4.1 JNK-mediated ROS in apoptosis signaling

JNK is well-known to promote cell death, as well as cell survival. However, the proposed mechanisms underlying JNK-dependent apoptosis are still controversial, depending on stimuli- and cell-types, possibly due to the periodic activation of JNK signaling. Prolonged JNK activation at the initial stage seemed to promote survival of the cells, indicating a pro-survival row of JNK. Meanwhile, a sustained activation of JNK can promote cell death (Weston and Davis, 2007), thus indicating that JNK is an important signaling pathway in cellular response to stress.

It has been generally known that ROS are potent inducers of JNK activation, as most studies on ROS-induced JNK activation results from the usage of H_2O_2 (Torres, 2003). Enhanced production of ROS is usually observed in cells exposed to ultraviolet (UV) radiation (Chan et al., 2003; Ding et al., 2002), ionizing radiation (Choi et al., 2007; Morales et al., 1998), cancer

chemotherapeutics (Nebbioso et al., 2011; Policastro et al., 2009) and TNFα (Deng et al., 2012; Kim et al., 2010).

At present, one of the most extensively studied pathway for ROS mediated JNK activation is via the TNF signaling, TNFR1 (Shen and Pervaiz, 2006; Takeda et al., 2003; Torres, 2003). TNFα stimulation leads to two distinct activation pathways: 1) earlier and transient activation of JNK is mediated by TRAF2 (Lamb et al., 2003) while the delayed and persistent JNK activation is mediated by ROS (Sakon et al., 2003; Ventura et al., 2004). Several studies have showed that the accumulation of ROS promotes prolonged JNK activation through JNK phosphatases inactivation and subsequently, TNF-mediated cell death (Kamata et al., 2005; Kim et al., 2007b; Micheau and Tschopp, 2003).

In mitochondria, ROS-mediated apoptosis seem to generate from a few pathways. One well-known example is through NADPH oxidase 1 (Nox1) (Hamanaka and Chandel, 2010). TNF-induced ROS is mainly generated by reduced Nox1 and Rac1, which are recruited to complex I by associating with TRADD, RIP1 and mitochondrial respiratory complex I (Seki et al., 2012). JNK seems to amplify mitochondria mediated ROS production during stimuli, causing JNK to translocate to mitochondria upon mitochondria stress, subsequently leading to higher amount of ROS produced through complex I formation (Chambers and LoGrasso, 2011). One recent example is the role of Apaf-1 in mediating ROS-induced apoptosis, in which the deficiency of Apaf-1, an important member of the apoptosome, significantly abrogates all UV-induced signal deterioration and cell death in mouse embryo fibroblasts (MEFs) (Feng et

al., 2012). Meanwhile, the ROS modulator 1 (Romo1) and Bcl-xL are recently discovered to be important regulators of TNF-induced ROS (Kim et al., 2010), as the complex II components seem to bind to the C-terminus of Romo1 located in the mitochondria, allowing the recruitment of Bcl-xL by Romo1. Subsequently, MOMP is reduced and ROS is increased and finally leading to apoptosis (Kim et al., 2010).

NF-κB exists as homodimers or heterodimers of the Rel proteins, which are characterised by the possession of a Rel homology domain that is required for specific binding, dimerization and nuclear localization. NF-κB is normally sequestered in the cytoplasm by the inhibitor IκB. Stimulation then leads to the activation of IKK, causing IκB to be phosphorylated, ubiquitinated and degraded to liberate the NF-κB dimers. This thus enables the freed NF-κB proteins for nuclear translocation and binding to specific target genes for transcriptional activity (Shen and Pervaiz, 2006). TNF-induced ROS accumulation and prolonged JNK activation seem to be suppressed by NF-κB-mediated sequestration of ROS and the induction of cFLIP (Chang et al., 2006; Kamata et al., 2005; Pham et al., 2004).

TNF-mediated activation of JNK1 phosphorylates and stimulates the activity of E3 ubiquitin ligase Itch, which is involved in the K48-linked ubiquitination of cFLIP to promote apoptosis via activation of caspase-8 and caspase- 3 in hepatocytes. The depletion of Itch prevents the ubiquitination and degradation of cFLIP that subsequently abrogates liver injury mediated by TNF (Chang et al., 2006). Nevertheless, different roles of JNK1 and JNK2 have been

proposed, as JNK1 disruption protected hepatocytes from TNF-induced death (Chang et al., 2006) while JNK2-deficient hepatocytes have a higher activity of TNF-induced JNK1 activity as well as a higher anti-apoptotic Mcl-1 protein level (Kodama et al., 2009). JNK1 seems to be pro-apoptotic, but JNK1 might serve to be anti-apoptotic via stabilization of Mcl-1 protein in JNK2-deficient cells (Kodama et al., 2009).

The role of JNK mediated ROS in apoptosis is also essentially mediated by the upstream MAPKKK, ASK1. ASK1 activity is first showed to interact with thioredoxin, an important cellular redox regulator, in which the reduced form of thioredoxin is able to bind to ASK1 to block its kinase activity. ROS stimulated oxidizes thioredoxin, thus causing its dissociation from ASK1 and subsequently ASK1 is activated through an essential threonine residue in the activation loop (Gotoh and Cooper, 1998; Saitoh et al., 1998; Tobiume et al., 2001). Thioredoxin also promotes ASK1 ubiquitination and degradation, thereby inhibits ASK-1 mediated JNK activation and apoptosis (Liu and Min, 2002). Meanwhile, the Kelch domain containing 10 (KLHDC10), which is the mammalian ortholog of Slim, a kelch protein that inactivates ASK1 in *Drasophila*, is found to interact with protein phosphatase 5 (PP5) to suppress ASK1 activation in response to ROS (Sekine et al., 2012).

On the other hand, other mediators are also important regulators of the JNK signaling in ROS-induced apoptosis. The TRAIL receptors, death receptors 4 (DR4) and 5 (DR5) are also involved in mediating JNK and ROS signaling, as observed in tumour cells studied recently (Park et al., 2012). Upon stimuli, DR4

and DR5 recruits riboflavin kinase (RFK) causing the activation of Nox1 at the intracellular death domains and this DR4/DR5-RFK complex is subsequently stabilised by TRADD to promote apoptosis (Park et al., 2012). Another example is the CD95 ligand (CD95L) that acts through the EGF receptor (EGFR) signaling; in which activated EGFR enhances the activity of CD95L to promote ROS production, JNK activation, FADD and caspase- 8 recruitment followed by apoptosis in rat hepatocytes (Reinehr et al., 2005). Meanwhile, the role of MLK3 in positively regulating ROS-mediated JNK activation and cell death is also seen in another study. MLK3 mutant protected cells from JNK activation, reduced MOMP release as well as apoptosis, signifying the MAPKKK function in the regulation of ROS upstream of mitochondria and JNK signaling (Hong and Kim, 2007). In addition, another mediator includes the Src kinase activity (Yoshizumi et al., 2000), which possibly binds to the docking protein of EGF signaling, Gab1 in promoting JNK activation (Chen et al., 2001).

1.4.4.2 JNK-mediated ROS in necrosis

As compared to oxidative stress mediated apoptosis, the studies on ROS in necrosis is much less understood at present. It is generally believed that ROS are capable in causing either apoptosis or necrosis, depending to the extent of the stimuli, the cell type and the signaling pathway triggered in response to ROS (Gardner et al., 1997; Shen and Liu, 2006; Troyano et al., 2003).

In TNF-induced cell death, the activation of Nox1 is also essential in mediating necrotic cell death. Upon stimuli, RIP1 recruits Nox1 to mediate a signaling complex consisting TRADD, RIP1, Nox1, and the small GTPase Rac1

(Kim et al., 2007b). In that study, RIP1 deficient cells are unable to form the signaling complex whereas Nox1 inhibition prevented necrotic cell death in MEFs in TNF-induced necrotic signaling, thus signifying an additional role of Nox1 in promoting necrosis (Kim et al., 2007b), apart from apoptosis as discussed earlier. Meanwhile, MLKL was recently discovered to be an essential player in TNF-induced necrotic cell death downstream of RIP3. MLKL seems to be required for ROS generation as well as the later phase of JNK activation, while MLKL inhibition blocks necrosis in human colon adenocarcinoma HT-29 cells (Zhao et al., 2012).

TRAF2 seems to be important in the regulation of ROS-induced necrosis, as H_2O_2-induced JNK activation is significantly impaired in MEFs deficient of RIP and TRAF2 (Shen et al., 2004). Moreover, cell death ligands such as FasL and TNFα are able to induce caspase-independent or necrotic cell death. This was first observed in murine L929 fibrosarcoma cells (Vercammen et al., 1998a; Vercammen et al., 1998b). Other studies have also showed similar observation, evidenced by the presence of necrotic cell death in other cells such as fibroblasts and human T lymphocytes (Lin et al., 2004; Sakon et al., 2003; Ventura et al., 2004). Moreover, in the MEF cells deficient of NF-κB ($p65^{-/-}$), TNFα treatment enhances ROS production to mediate sustained JNK activation and subsequently, necrotic cell death (Kamata et al., 2005; Sakon et al., 2003). Also, JNK may mediate PARP-induced necrotic cell death by direct phosphorylation and activation of PARP-1, as JNK ablation or inhibition suppresses PARP-1 activation and caspase-independent cell death (Zhang et al., 2007). More recently,

the role of TAK1, a MAPKKK, has been implicated in regulating necroptosis as well as apoptosis, as TNFα-induced cell death in TAK1-deficient MEFs do not undergo cell death when simultaneous inhibition of caspases and knockdown of RIP3 are performed (Lamothe et al., 2012). Nevertheless, the role of JNK in ROS-mediated necrosis remains elusive at present.

1.5 Objectives of the study

At present, the role of autophagy and cell death regulation as well as its manipulations for therapeutic benefits in the pathogenesis of TSC has yet to be fully explored. As it has been previously reported that TSC-deficient cells are with constitutively active mTORC1 and hypersensitive to various cell death inducers, the main objective of this thesis is to uncover the functional roles of TSC in regulation of autophagy and cell death. Thus, to achieve this objective, we aimed to conduct the following two aspects of study:

1. To investigate the role of TSC in the regulation of autophagy in response to starvation conditions and,
2. To study the role of TSC in oxidative stress mediated JNK signaling and cell death

The present study identifies a novel role of TSC in autophagy and cell death as well as its roles in MAPK-JNK signaling, which may be implicated in the pathological functions of TSC-related disease. It is thus hoped that results from this study could contribute for the therapeutic benefits of TSC-related pathology.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell lines and cell culture

TSC2$^{+/+}$-*p53*$^{-/-}$, TSC2$^{-/-}$-*p53*$^{-/-}$, WT-*p53*$^{+/+}$ and TSC1$^{-/-}$-*p53*$^{+/+}$ mouse embryonic fibroblasts (MEFs) were generous gifts from Dr. David J. Kwiatkowski (Brigham and Women's Hospital, Boston, MA) and were described previously (Kwiatkowski et al., 2002; Zhang et al., 2003a), *p53*$^{-/-}$ MEFs of reconstituted TSC2, TSC2$^{-/-(+/+)}$ and pairing TSC2$^{-/-}$ with empty vector (EV) were kind gifts from Dr. Huang Jingxiang (National University Hospital, Singapore) and Dr. Brendan D. Manning (Harvard School of Public Health, Boston, USA) that was described earlier (Huang et al., 2008). Atg7$^{+/+}$ and Atg7$^{-/-}$ MEFs were generous gifts from Dr. Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science, Japan). 293FT cells were purchased from Invitrogen. All cells were cultured up to passage-10 in DMEM (Sigma) supplemented with 10% FBS (Hyclone), 1% penicillin-streptomycin (Invitrogen) and maintained in a 5% CO_2 incubator at 37 °C.

2.2 Reagents and antibodies

All the following antibodies were purchased from Cell Signaling: Akt (#9272), phospho-Akt (#9271), p70S6K (#9202), phospho-p70S6K (#9205), caspase 3 (#9662), PARP-1(#9542), raptor (#2280), TSC2 (#3612), TSC2 (#3990), 4EBP1 (#9644), phospho-4EBP1 (#2855), phospho-c-Jun (#9261), c-Jun (#9165), TSC1 (#4963), phospho-MKK3/MKK6 (#9236), phospho-p38 (#9211), phospho-MKK4 (#4514), phospho-MKK4 (#9151), p38 MAPK (#9212), and phospho-threonine (#9386). Anti-p62 (H00008878-M01) was obtained from Abnova while Apg7 (#3617) was purchased from ProSci. The reagents: rapamycin (#R0395), leucine

(#L8912), insulin-like growth factor 1 (IGF-1, #I8779), Earles's balanced salt solution (EBSS #E2888), chloroquine diphosphate (CQ, #C6628), PP242 (#P0037), sodium nitroprusside (#431451), sodium orthovanadate (#S6508), and okadaic acid (#O4511); while anti-LC3 antibody (#L7543), anti-α-tubulin (#T6199), anti-β-actin (#A5441), anti-HA (#H3663), and anti-Flag (#F3165) antibodies were purchased from Sigma-Aldrich. Necrostatin-1 (#480065) was from Merck. Phospho-JNK was from Biosource (#44-682G). Anti-MKP-1 (#SC-370) and phospho-tyrosine (#SC-7020) were from Santa Cruz. Goat anti-rabbit (#31460) or anti-mouse (#31430) horseradish peroxidase-linked antibodies were used as secondary antibodies. The phospho-ASK1 was a generous gift by Dr. H. Ichijo (University of Tokyo, Japan) described earlier (Tobiume et al., 2002).

For the preparation of antibodies, 0.5 g of bovine serum albumin (#A9418, Sigma) was weighed and dissolved in 10 ml of 1 X Tris Buffered Saline with Tween 20 (TBST) to make up a 5% BSA and 0.01% NaN_3 final solution. NaN_3 was added to prevent bacterial contamination. All antibodies prepared were stored at 4°C. A 1:1000 dilution for all antibodies were used unless indicated as follow; 1:2000 dilution for p-ASK1 and p-JNK, respectively; 1:5000 dilution for anti-p62, anti-actin and anti-tubulin.

2.3 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

For MTT assay, 5 mg/ml of working solution was prepared in 1 X PBS. Cells were plated in 96-well, leaving some wells were unseeded and left as blanks for control. After designated treatments, 20 µl of MTT solution was added into

control (blanks, with medium only) and treated wells. The plate was incubated for 2 hours in the incubator (37°C, 5% CO_2), for the yellow MTT to be solubilised into formazon by the mitochondria of living cells. Medium was then aspirated and formazon was resuspended in 200 µl DMSO with 10 seconds shaking. Optical density was obtained at 590 nm.

2.4 Propidium iodide (PI) live exclusion staining for cell viability

For PI staining, the medium in each well was collected and cells were harvested with trypsin after treatments. Then, cell pellets obtained were resuspended in 1X phosphate buffer saline (PBS) containing PI at a final concentration of 5 µg/ml and incubated for 10 minutes at 37°C. Ten thousand cells from each sample were analysed with FACSCalibur flow cytometry (BD Bioscience) using CellQuest software.

2.5 Transient siRNA transfection

Cells were transfected with 100 nM of either the non-specific control, mouse Atg7, raptor, or TSC2 siRNA (ON-TARGET plus SMARTpool^{TM}), respectively by using DharmaFECT^{TM} 4 siRNA Transfection reagent (Dharmacon) according to the manufacturer's protocol. After 48 hours of siRNA transfection, cells were seeded overnight for 70% confluency prior to designated treatments on the next day.

2.6 RNA extraction

Total RNA was isolated from cell cultured, according to designated treatments using RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. The concentration and quality of total RNA was determined using NanoDrop 1000

Spectrophotometer (Thermo Scientific). Extracted RNAs were stored at -80°C.

2.7 Reverse transcriptase polymerase chain reaction (RT-PCR)

The complementary DNA (cDNA) was synthesised with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. An amount of 0.6 µg RNA was reverse transcribed into cDNA in a 10 µl reaction, consisting of RT Buffer, dNTPs, random primers and reverse transcriptase that are incubated for 10 minutes at 25°C, 37°C for 2 hours and subsequent 85°C for 5 minutes to inactivate the enzyme used (MyCycler^{TM} Thermal Cycler, Bio-Rad). Products obtained from RT-PCR were diluted 1:5 times and subsequently used in quantitative real-time PCR (qPCR).

2.8 Quantitative real-time PCR (qPCR)

The qPCR was carried out in a thermal cycler (model C1000^{TM}, Bio-rad) using SsoFast EvaGreen (#1725201AP, Bio-Rad) in a final volume of 10 µl reaction containing 2 µl of synthesised cDNA obtained from RT earlier as template, 0.5 µM of each forward and reverse primers, 5 µl of SsoFast master mix and remaining with RNAse-free water (2 µl). *GAPDH* were used as internal control. Three independent experiments were performed. Samples were normalised to the levels of *GAPDH* and subsequently, data was analysed using CFX^{TM} Manager software (Bio-Rad). All the MKPs primers (MKP-1, -3, -5, -7) listed in Table 2.1 were used for qPCR analysis. The MKP primers were generous gifts from Dr. Zhang Yongliang (National University of Singapore, Singapore).

2.9 Plasmids and transient transfection

The plasmids used in this thesis were kind gifts from the following parties:

Table 2.1 Primers sequence of genes for mRNA analysis

Name	Direction	Sequence (5' to 3')
MKP-1	Forward	CTTTCTCCAAGGAGGATATGAA
	Reverse	CTTGATGGAGTCTATGAAGTCA
MKP-3	Forward	ATAGATACGCTCAGACCCGTG
	Reverse	CTGAAGCCACCTTCCAGGTA
MKP-5	Forward	GCTGTCCACATTAACTGTGCCG
	Reverse	TCTCTTCAGGGACTCGAGGA
MKP-7	Forward	ATGGAGTGGCTGAGCCTTTG
	Reverse	CGGCTATCAATTAGCAGCACT
GAPDH	Forward	GCACAGTCAAGGCCGAGAAT
	Reverse	GCCTTCTCCATGGTGGTGAA

pcDNA3-HA-TSC2 (rat) (Dr. Guan Kun-Liang, University of California, San Diego, USA) (Inoki et al., 2002), pEF6-TSC2 (human) was from Dr. David J. Kwiatkowski (Boston, USA) (Zhang et al., 2003a), pSRα-3HA-JNKK2-JNK1 construct was from Dr. Lin Anning (University of Chicago, Chicago, USA) and Flag-MKP-1 was from Dr. Zhang Yongliang (National University of Singapore, Singapore). Overexpression studies were performed according to manufacturer's protocol using Lipofectamine^{TM} 2000 (#11668-019, Invitrogen). Cells were transfected with recommended amount (μg) of plasmid for 48 hours, and reseeded to reach cell confluency of 70% prior to designated treatment after overnight incubation. pLenti4-TSC2 construct was also used in the transient transfection in subsequent studies.

2.10 DNA extraction

The plasmids were extracted using Qiagen Miniprep Plasmid Extraction Kit and Qiagen Maxiprep Plasmid Extraction Kits, according to the volume of DNA required in the study. For the smaller amount of DNA extraction, the Miniprep kit was used while the Maxiprep kit was utilised for larger volumes of DNA

extractions. The bacterial plasmid cultured was first centrifuged to obtain the cell pellet, while the supernatant was discarded. Bacterial cell pellets were then suspended in a series of buffers provided in the respective extraction kits, according to the manufacturer's protocol.

2.11 Immunoprecipitation

For immunoprecipitation (IP), cells were lysed in lysis buffer (50 mM HEPES pH 7.4, 1% NP-40, 150 mM NaCl, 5% glycerol, 1 mM EDTA) freshly supplemented with protease inhibitor (Roche) and phosphatase inhibitors (Halt) prior to usage. After designated respective treatment, the medium was aspirated and cells were washed with 1 X cold PBS. PBS was removed and cells were subsequently scraped in 1 ml of IP lysis buffer. Cell extracts were lysed and rotated for 30 minutes at 4°C and subsequently spun at the highest speed with a microcentrifuge for 10 minutes. A partial amount of supernatant was kept as total cell lysates (100 µl). The remaining supernatant collected was pre-cleared with protein A/G agarose beads for 1 hour at 4°C prior to the addition of 1-2 µg of antibodies and incubated overnight. On the following day, cell lysates were added with protein A/G agarose (#20422, Thermo Scientific), rotated for 2 hours at 4°C, and then extensively washed with lysis buffer for 3 times. After spinning down in a microcentrifuge, the precipitates obtained were eluted in Laemmli loading buffer, boiled for 5 minutes and retained further for SDS-PAGE analysis.

2.12 Western blotting

After the indicated time of designated treatments, cells were lysed in M2 lysis buffer (20 mM Tris at pH 7, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM

EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM glycerol phosphate, 1 mM sodium vanadate and proteinase inhibitor cocktail). An equal amount of protein was resolved by sodium SDS-PAGE and transferred onto PVDF membrane (Bio-Rad). After blocking with 5% non-fat milk, the membrane was probed with designated first and second antibodies, developed with the enhanced chemiluminescence method (Pierce and Merck) and visualised using Kodak Image Station 440CF (Kodak) and ImageQuant LAS500 (GE Healthcare). Quantification results for the western blots were obtained using Kodak 1D 4.5.1 software. We further modified the lysis buffer used in subsequent studies (in Chapter 4), using sodium dodecyl sulfate (SDS) Laemlli lysis buffer consisting of 2% SDS, 20% glycerol, 1 M of Tris hydrochloric acid, 2 mM dithiothreitol, Halt Phosphatase Inhibitor and Roche Protease Inhibitor.

2.13 Establishment of TSC2 reconstitution in stable cell line

For the construction of stable *TSC2* into $TSC2^{-/-}$ MEFs, the following steps were involved, which were: i) generation of an entry vector, pENTR™/D-TOPO-TSC2, ii) generation of the destination vector, pLenti4/TO/V5-DEST-TSC2, iii) construction of constitutive, stable expression of wild type *TSC2* in $TSC2^{-/-}$ MEFs using lentivirus infection, followed by iv) effects of reconstituted *TSC2* expression studies using immunoblotting.

2.13.1 Generation of entry vector pENTR™/D-TOPO-TSC2

2.13.1.1 PCR amplification of *TSC2* DNA

Prior to DNA amplification from the pEF6-TSC2 plasmid, the primers in Table 2.2 were first used to verify the full length of *TSC2* gene through sequencing.

Table 2.2 TSC2 primers used for *TSC2* reconstitution

Name	Direction	Sequence (5' to 3')
start	Forward	CACCATGGCCAAACCAACAAGCAAAGAT
stop	Reverse	TCACACAAACTCGGTGAAGTCCTCAC
731-sequence	Forward	ACCGCGTCCTCTGTGGACAT
1351-sequence	Forward	GGACCAGAGGCCTGAGTCCT
1971-sequence	Forward	GGGCCGACTCACTGCACCGC
2591-sequence	Forward	AAGCTCACGCACATCTCAGC
3211-sequence	Forward	CTCCAACTTCACGGCTGTCC
3831-sequence	Forward	AGGAGCACCGGGACACAGCC
4451-sequence	Forward	GGCAAGAGAGTAGAGAGGGA
5071-sequence	Forward	CGAGTGCAACCTGGTGTCCC

After verification, the full-length cDNA of *TSC2* was isolated using PCR using the following cycle for a 20 µl reaction (reaction contained DNA, dNTPs, reaction buffer, sterile water, polymerase enzymes and a pair of TSC2 start and stop primers as indicated in Table 2.2): i) 94°C for 3 minutes to initialize enzymes, ii) 94°C for 45 seconds, iii) 50°C for 45 seconds, iv) 72°C for 1 minute , followed by 30 repeating cycles from step (ii) to step (iv) for the series of denaturation, annealing and elongation, respectively. Final elongation was subsequently performed by heating the samples to 72°C for 7 minutes. Products were kept temporarily at 4°C prior to usage. The start codon primer of TSC2 was designed to contain the 'CACC' in the end of the 5' forward primer to form base pair with the 'GTCC' overhang sequence in the pENTR™ TOPO vector (Table 2.2).

2.13.1.2 Bacterial plasmid transformation

The PCR products of *TSC2* were first cloned into pENTR™/ D-TOPO vector to generate an entry vector (pENTR™/D-TOPO-TSC2), using pENTR™

Directional TOPO Cloning Kit (Invitrogen). The cloning reaction was prepared by mixing salt and TOPO vector (both supplied by the kit), with sterile water and 2 µl of fresh PCR products of *TSC2*. The reaction was incubated for 5 minutes at room temperature followed by placing the tube on ice for 30 minutes of incubation. Meanwhile, the following materials were prepared: i) water bath was warmed to 42°C, ii) S.O.C medium supplied in the kit was warmed to room temperature, iii) two selective kanamycin (50 µg/ml) agar were warmed to 37°C and finally, iv) a vial of One Shot Top10 (Invitrogen) *Escherichia coli* competent cells was thawed in ice for bacterial transformation. After the stipulated incubation time, the cloning reaction prepared earlier was added into the thawed Top10 *E. coli* cells. The suspension was mixed gently without pipetting, followed by 20 minutes incubation on ice. After the incubation time, the cells were heat-shocked for 30 seconds at 42°C without shaking. The tube was then immediately transferred to ice and added with 250 µl of S.O.C. medium and placed on a shaker (200 rpm) at 37°C for 1 hour. Subsequently, 50-200 µl of each transformation was spread on two different kanamycin selective plates. The plates were incubated overnight at 37°C.

2.13.1.3 PCR colony analysis

On the following day, about 10 single colonies were picked for PCR colony analysis. Basically, the colonies were resuspended individually in 50 µl of PCR SuperMix containing *TSC2* forward and reverse primers, as well as M13 forward and M13 reverse primers that were supplied in the kit. M13 primers were needed to aid in the sequencing of intended gene in the entry vector used, as the entry

plasmid contains a region of M13 bacteriophage priming site needed for cloning. The reaction was first incubated for 10 minutes at 94°C to lyse cells and inactivate nucleases. Then, the cycle was amplified for 30 cycles and final extension was performed by incubating the products at 72°C for 10 minutes. Products was stored at 4°C and subsequently used for agarose gel electrophoresis visualization. The same 10 colonies were also streaked on a kanamycin selective agar plate to make a patch plate to preserve the colonies for further analysis.

2.13.2 Generation of destination vector pLenti4/TO/V5-DEST-TSC2

2.13.2.1 Identification and preservation of positive clone

Following verification of the 10 single colonies contained in the entry vector through agarose gel visualization, the *TSC2* DNA from a positive colony was extracted and analysed by gene sequencing to verify the correct gene insertion. The positive clone was also preserved in a glycerol stock, which was prepared by mixing 0.85 ml of culture containing stationary phase of bacterial stock with 0.15 ml (15%) of sterile glycerol in a cryovial. The glycerol stock was subsequently stored in -80°C.

2.13.2.2 LR Recombination Reaction

LR recombination reaction was performed to generate the destination vector of pLenti4/TO/V5-DEST-TSC2 (Figure 2.1). To prepare the LR recombination reaction, purified plasmid containing *TSC2* of the entry clone (50-150 ng) was added into a mixture of reaction that contained pLenti4/TO/V5-DEST vector (Figure 2.1), TE Buffer, Proteinase K, and LR Clonase^{TM} II Enzyme Mix. The reaction was incubated for 10 minutes at 37°C. Meanwhile, a vial of One Shot

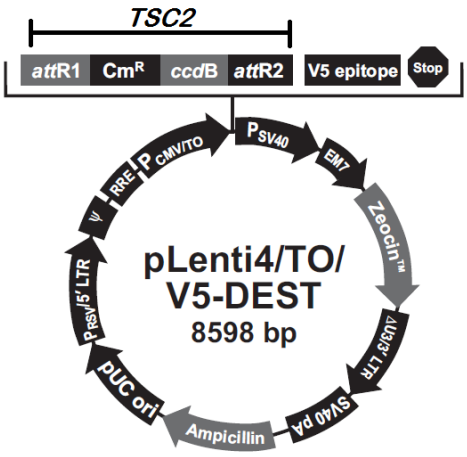


Figure 2.1 Map of expression for vector pLenti4/TO/V5-TSC2.

Stbl3TM competent cell was thawed on ice and the prepared LR reaction was subsequently added to the cells. Transformation was performed using ampicillin-resistant agar plates. Expression clone was propagated, preserved in glycerol stock and plasmid extraction was subsequently performed using Qiagen Maxiprep Extraction kit.

2.13.3 Construction of constitutive, stable expression of wild type *TSC2* in TSC2$^{-/-}$ MEFs using lentivirus infection

2.13.3.1 DNA purification

Purified plasmid DNA used for transfection must be free from contamination. Therefore, pLenti4/TO/V5-TSC2 obtained was purified by adding equal volume of phenol:chloroform:isoamyl alcohol 25:24:1 (#P3803, Sigma). The tube was then gently vortexed and subsequently spun for 5 minutes at highest speed (~16 000 x *g*). The upper aqueous phase containing DNA was transferred cautiously into a new tube, followed by the addition of 2.5 X volume of 100% pure ethanol. Then, 10% of 10 M ammonium acetate was added and the suspension was gently

mixed. The tube was subsequently incubated overnight at -20°C. On the following day, the tube was spun in a microcentrifuge for 20 minutes at the highest speed (~16 000 x *g*). DNA pellet obtained was then added with 70% ethanol to wash the pellet, followed by centrifugation for 2 minutes at 16 000 x *g*. Supernatant was discarded gently and the DNA pellet was air-dried. Purified DNA was then dissolved in double distilled water and stored in -20°C for further usage.

2.13.3.2 Lentivirus production in 293FT cells

The expression plasmid needed for each transfection was 3 µg. For the generation of lentiviral stock, low passages of 293FT cells were seeded in 10 cm^3 dishes in antibiotics-free medium and incubated overnight, with at least 90% confluency (6 x 10^6 cells) for transfection on the following day. DNA-Lipofectamine™ 2000 complex was prepared, in which the first tube contained 9 µg of Virapower™ Packaging Mix, 3 µg of pLenti4-TSC2 in 1.5 ml of Opti-MEM® I medium without serum. In another sterile tube, 36 µl of Lipofectamine™ 2000 was mixed into 1.5 ml of Opti-MEM® I medium without serum. Both tubes were mixed gently and incubated for 5 minutes at room temperature. Then, both solutions were mixed together and further incubated for another 20 minutes. While DNA-lipid complexes were forming, 293FT cells were trypsinised and and counted to form a density of 1.2 x 10^6 cells/ ml in growth medium containing serum. The DNA-lipid complexes were added to a sterile 10 cm^3 culture plate containing 5 ml of antibiotics-free growth medium with serum, followed by the addition of 5 ml of 293FT cells (6 x 10^6 total cells). After gentle mixing, the plate was placed in a CO_2 incubator at 37°C. On the following day, medium was changed to DMEM

with additional 1 mM sodium pyruvate. The virus-containing supernatant was harvested 72 hours later into a 15 ml centrifuge tube and centrifuged at 3000 rpm, 4°C for 5 minutes to pellet cell debris. Viral supernatants were pipette into cryovials in 1 ml aliquots and stored at -80°C.

2.13.3.3 Transduction of virus with TSC2$^{-/-}$ MEFs

Low-passage TSC2$^{-/-}$ MEFs were plated in a 10cm^3 such that the cells would be 50-60% confluent on the day of virus transfection. Lentiviral stock was filtered prior to cell infection. After removing culture medium from the plated cells, virus-containing medium was added by gentle resuspension into the cells. Polybrene® (#H9268) was additionally added to a final concentration of 6 µg/ml. The medium was removed on the following day (day 2) and cells were replaced with fresh complete medium. On day 3, cells were trypsinised, replated to reduce cell density and medium was replaced with Zeocin™ (100 µg/ml) (#R250-01, Invitrogen) containing medium. Medium was changed every 2 to 3 days until about 2 weeks later, when Zeocin™-resistant colonies could be identified. At least 30 colonies were selected, expanded and preserved. Clones of cells chosen were subsequently verified for reconstituted *TSC2* expressions through immunoblotting and used for subsequent studies.

2.14 Statistical analysis

The image data were representatives from at least three repeated experiments. All numeric values were expressed as mean ± SD from at least three independent experiments. The *p*-value was calculated using Student's *t*-test with *p*-values<0.05 (**p*<0.05) or *p*-values<0.01 (***p*<0.01) is considered to be statistically significant.

CHAPTER 3

IMPAIRED AUTOPHAGY DUE TO CONSTITUTIVE mTOR ACTIVATION SENSITIZES TSC2-NULL CELLS TO CELL DEATH

3.1 Introduction

Autophagy, or 'self-eating' is an catabolic process in which cytoplasmic constituents including macromolecules (such as proteins, glycogens, lipids and nucleotides) and organelles (such as mitochondria, peroxisomes and endoplasmic reticulum) are degraded by the hydrolytic enzymes in autolysosome and finally the recycling of the resulting macromolecules (Yang and Klionsky, 2010a). At present, the biological functions of autophagy have been well-studied. Autophagy is vital in maintaining cell and tissue homeostasis via clearing intracellular misfolded proteins and damaged organelles, in ageing prevention, in pathogen elimination, in cell death, and in tumour suppression (Mizushima, 2005; Yang and Klionsky, 2010a). Among them, one important aspect is that autophagy mediates cell survival under starvation conditions, in which cells recycle cytoplasmic constituents through lysosomal degradation to provide the essential nutrients for survival (Yang and Klionsky, 2010a). The cytoprotective roles of autophagy against death have also been showed in tumour growth under limited nutrients and oxygen conditions (Fung et al., 2008) as well as in drug-induced cell death in cancer cells (Amaravadi et al., 2007).

Autophagy is a dynamic process that involves the following consecutive steps: 1) initiation, 2) nucleation, 3) elongation and 4) fusion and maturation processes. These processes are known to be controlled by a group of proteins encoded by Atg. For instance, the induction or initiation of autophagy is regulated via the ULK1/Atg1 complex downstream of mTORC1, while the nucleation process is mediated by the Beclin 1 and hVps34/class III PI3K complex (He and

Klionsky, 2009). In the membrane elongation and expansion step, a series of other Atgs are involved in two conjugation systems: Atg12-Atg5-Atg16 and LC3-PE (He and Klionsky, 2009). LC3 is largely cytosolic but exists as lipid-conjugated form and is localised to both sides of autophagosomes upon autophagy induction (Kabeya et al., 2000) and is widely used as an autophagy marker (He and Klionsky, 2009).

mTOR integrates diverse signals from growth factor, energy and nutrients network to act as a central regulator. Upon stimulation of growth factors signaling, mTOR is activated via the well-established PI3K-AKT-TSC pathway (Inoki et al., 2002; Potter et al., 2002). *TSC1* encodes hamartin (also known as TSC1) to form a heterodimeric functional complex with the gene product of *TSC2*, tuberin (known as TSC2) to act as GAP for Rheb. TSC2 stimulates the intrinsic GTP hydrolysis activity of Rheb by establishing the transition of Rheb from an active GTP bound to an inactive GDP-bound form (Garami et al., 2003; Zhang et al., 2003b). Upon binding to GTP, Rheb acts as a small GTPase directly activates mTORC1 (Long et al., 2005). On the other hand, amino acids can directly activate mTORC1 through Rag proteins binding to mTORC1 (Sancak et al., 2008). The activation of mTORC1 results in the phosphorylation of two downstream targets: S6K and 4EBP1 (eukaryotic translation-initiation factor 4E-binding protein) to trigger ribosome biogenesis and protein translation (Burnett et al., 1998). In contrast to the well-studied mTORC1, the signaling pathways and functions of mTORC2 are still unclear. One important function of mTORC2 is to activate Akt by phosphorylation (Sarbassov et al., 2005).

mTORC1 is probably the most important upstream negative regulator of autophagy. Under nutrients-rich conditions, mTORC1 suppresses autophagy initiation by inhibiting the ULK1/Atg1 complex by phosphorylating Atg13 and ULK1/2. Activated ULKs further stimulates autophagy induction by the phosphorylation and activation of Atg13 and the scaffold protein FIP200 and ULK1 itself. In contrast, starvation or rapamycin treatment causes the dephosphorylation and activation of ULK1/2 complex (Hosokawa et al., 2009; Jung et al., 2009).

Aberrant upregulation of mammalian target of rapamycin (mTOR) signaling pathway has been associated with various cancer signaling mechanisms. The loss of tumour suppressors such as PTEN, hamartin and tuberin, and STK11 (serine/threonine kinase 11; also known as LKB1) that activates mTOR pathway are linked to cancer, including bladder, lung, ovarian, melanoma, brain, endometrial, and thyroid; as well as hamartoma syndromes, such as tuberous sclerosis, Peutz-Jeghers syndrome, and Cowden disease (Guertin and Sabatini, 2005). TSC disease is a multisystem disease characterised by the formation of hamartomas in various organs (Crino et al., 2006). TSC disease is caused by autosomal dominant mutation in either *TSC1* or *TSC2*, although patients with *TSC2* mutations have more severe clinical findings than those with *TSC1* mutations (Sancak et al., 2005). Emerging evidence has showed that TSC-null cells, which are cells lacking either TSC1 or TSC2 and could not form a functional TSC1/2 complex, are more sensitive to cell death induced by various stimuli, as compared to its wild type counterparts. These stimuli include glucose

deprivation (Lee et al., 2007a), ER stress (Di Nardo et al., 2009; Kang et al., 2011), bacterial stimulated-inflammation (Weichhart et al., 2008), DNA damage and TNFα stimulation (Ghosh et al., 2006). The high susceptibility of TSC-null cells to death were reported to be due to increased p53 translation (Lee et al., 2007a), increased activation of Rheb to cause ER stress (Kang et al., 2011), lower NF-κB level (Ghosh et al., 2006), increased ER stress (Di Nardo et al., 2009; Ozcan et al., 2008), and defects in Akt activation (Huang et al., 2009a).

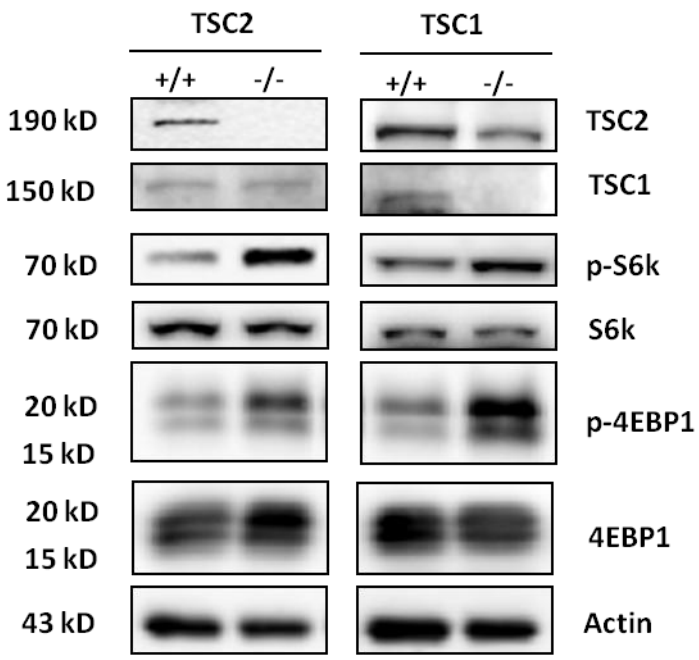
In this study, we attempted to study the involvement of autophagy in the enhanced sensitivity of TSC2-null cells to cell death stimuli. First, we confirmed that $TSC1^{-/-}$ and $TSC2^{-/-}$ cells are more susceptible to cell death induced by starvation and hypoxia. Second, we found that there is impaired basal and inducible autophagy in $TSC2^{-/-}$ cells. Finally, we demonstrated that autophagy plays a pro-survival role and the impaired autophagy due to mTOR hyperactivation contributes to enhanced cell death of $TSC2^{-/-}$ MEFs. Therefore, our findings reveal new insights into the complex relationships among mTOR, autophagy, and cell death.

3.2 Results

3.2.1 $TSC2^{-/-}$ MEFs are hypersensitive to apoptosis induced by various cell death stimuli

To explore the cell death sensitivity of TSC-null cells, the $TSC2^{+/+}$, $TSC2^{-/-}$ (both $p53^{-/-}$) as well as TSC1 MEFs (both TSC1 wild type and knockouts are $p53^{+/+}$) as shown in Figure 3.1A were treated with various death stimuli. There

A


TSC2
TSC1
+/+
-/-
+/+
-/-
190 kD
TSC2
150 kD
TSC1
70 kD
p-S6k
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S6k
20 kD
15 kD
p-4EBP1
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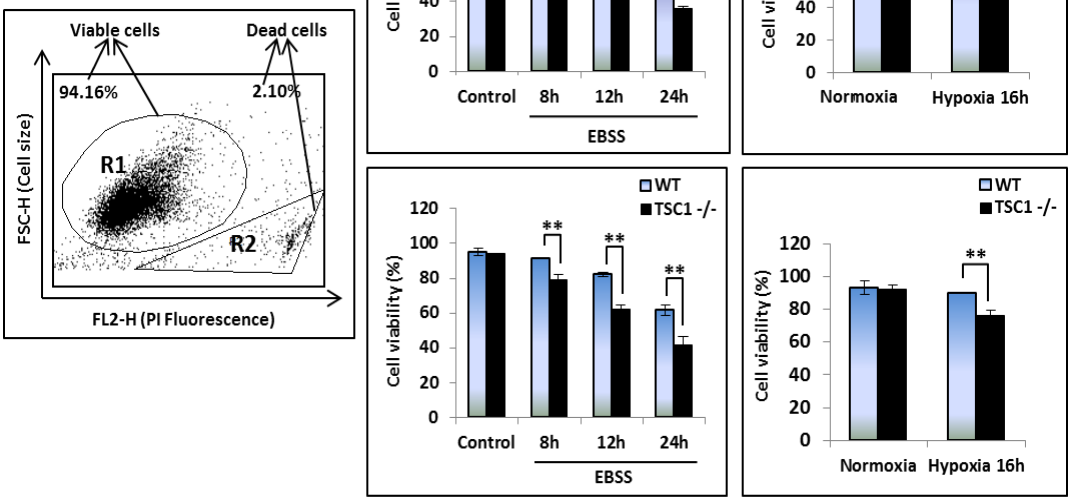
4EBP1
43 kD
Actin

B


Viable cells
Dead cells
94.16%
2.10%
R1
R2
FSC-H (Cell size)
FL2-H (PI Fluorescence)
TSC2+/+
TSC2-/-
Cell viability (%)
Control
8h
12h
24h
EBSS
TSC2 +/+
TSC2 -/-
Normoxia
Hypoxia 16h
WT
TSC1 -/-

C

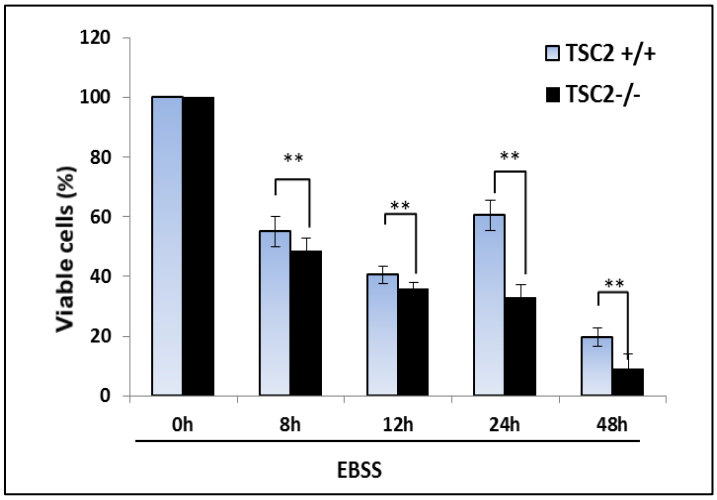


Figure 3.1 Statistical quantifications for the susceptibility of TSC cells induced by starvation and hypoxia stimuli. (A) TSC2- and TSC1-deficient cells. The $TSC2^{+/+}$ (wild type), $TSC2^{-/-}$ (TSC2 knockout) as well as the pair of $TSC1^{+/+}$ (wild type, WT) and $TSC1^{-/-}$ (TSC1 knockout) cells were collected after seeded overnight in full medium (DMEM). Cell lysates were used for western blotting and blotted for the indicated markers. Actin served as loading control. (B) Dotplot of propidium iodide (PI) live exclusion assay for quantification of viable and dead cells. The R1 and R2 regions in the dotplot represent viable and dead cells, respectively for control $TSC2^{+/+}$ cells treated in full medium of 24 hours (left panel). The $TSC2^{+/+}$, $TSC2^{-/-}$, WT, and $TSC1^{-/-}$ MEFs were treated with control (full medium) or EBSS (Earle's Balanced Salt Solution) (middle panel); and hypoxia (1% O_2, 5% CO_2, balance N_2) or normoxia (control, 21% O_2, 5% CO_2) (right panel) for the indicated time points. The percentage of viable cells was measured with PI (5 µg/ml) live exclusion staining. (C) MTT assay for the quantification of viable cells. $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were treated in EBSS as the indicated period of time. MTT stock (5 mg/ml) was added into the cells seeded in 96-well plates and results were analysed from optical absorbance obtained. All statistical data in all the figures are presented as means ± S.D of three independent experiments (**p<0.01, t-test).

was more significant cell death in TSC-null cells treated with starvation (with EBSS) and hypoxia, as quantified by PI exclusion test coupled with flow cytometry (Figure 3.1B) and MTT assay for starvation treatment (Figure 3.1C). The morphological changes as shown in Figure 3.2A were consistent with the PI exclusion test, showing more cell death in $TSC2^{-/-}$ cells treated with either EBSS

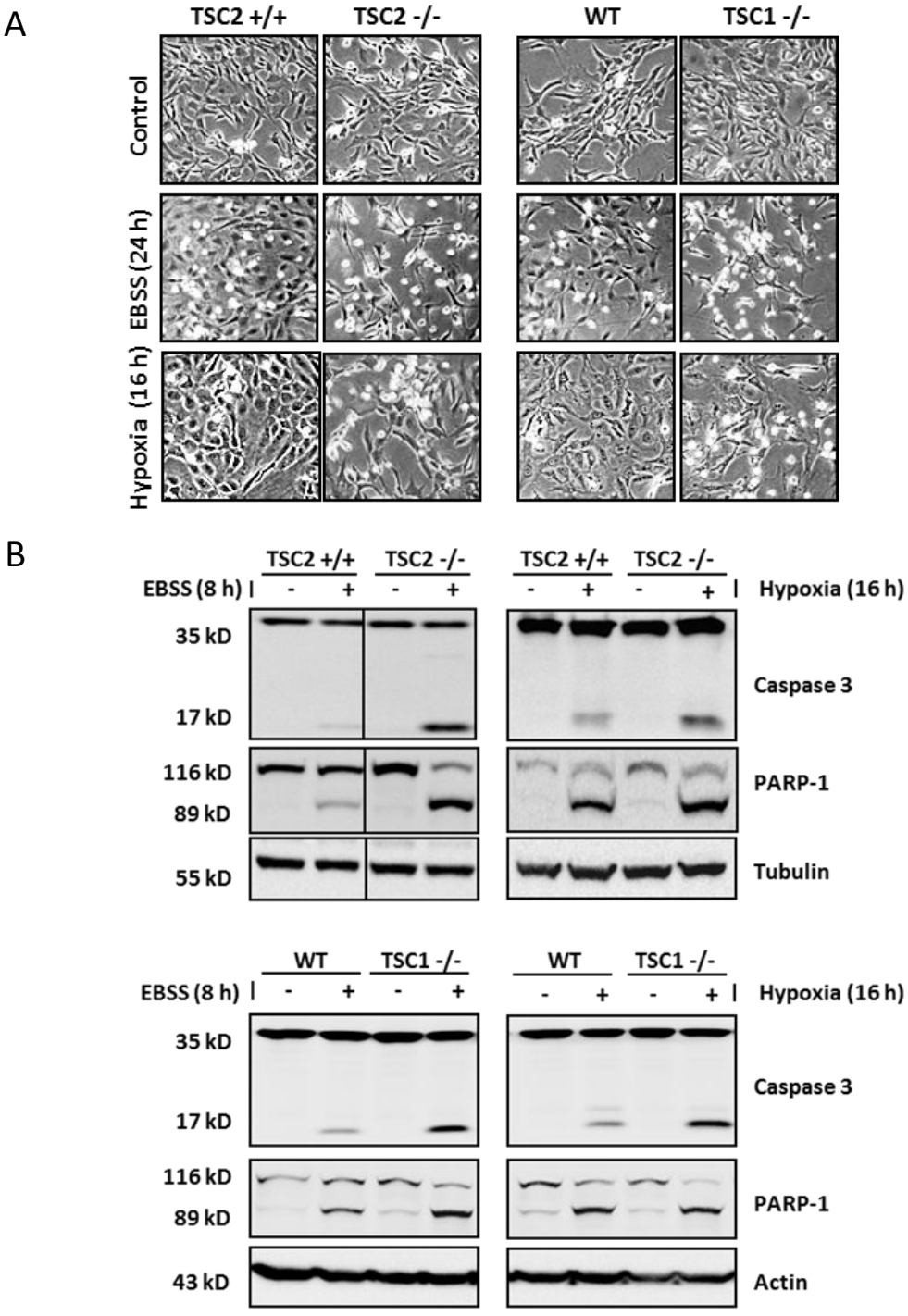


Figure 3.2 TSC-null cells are more sensitive to cell death induced by starvation and hypoxia stimuli. (A) Morphological changes of MEFs with respective treatments (as described in Figure 3.1). Cells were examined and photographed with an inverted microscope (X 100). (B) Cell lysates were analysed by western blotting using antibodies specific to caspase 3 and PARP-1 cleavages. Tubulin and actin served as loading control.

or hypoxia. Moreover, to examine the nature of cell death, we detected two apoptotic markers, caspase 3 and PARP-1 cleavages using western blot. As shown in Figure 3.2B (middle and lower panel respectively), there were evident cleavage of caspase 3 and PARP-1 in $TSC2^{-/-}$, but not in $TSC2^{+/+}$. Similar results were found with other oxidative stimuli including H_2O_2, sodium nitroprusside, as well as with TNFα (Figure 3.3). Cycloheximide was added into TNFα treatment to induce typical apoptosis while the pan-caspase inhibitor zVAD was used to block apoptosis (Figure 3.3B). As expected, TSC-null cells are more sensitive to apoptosis induced by various stimuli. Such observations are indeed consistent with some earlier reports on cell death in TSC-null cells (Di Nardo et al., 2009; Ghosh et al., 2006; Lee et al., 2007a; Kang et al., 2011).

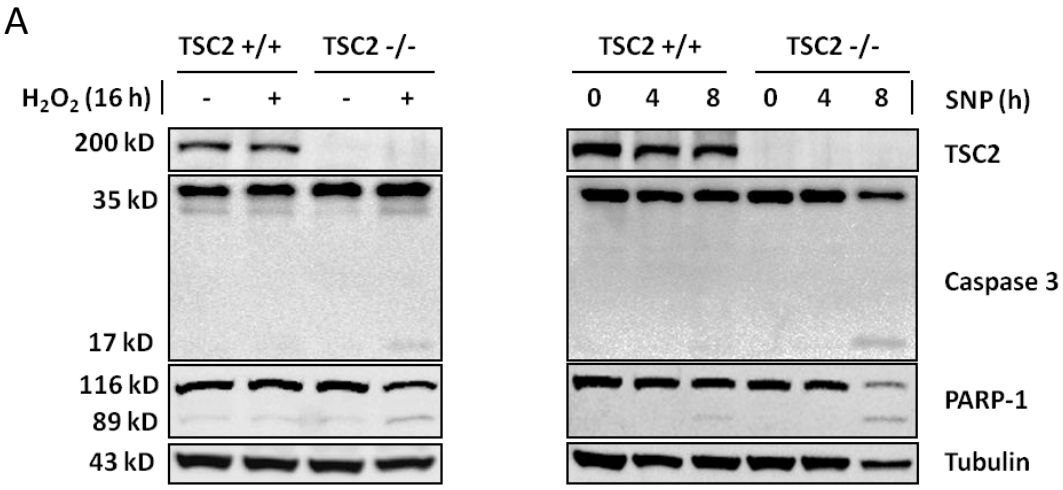
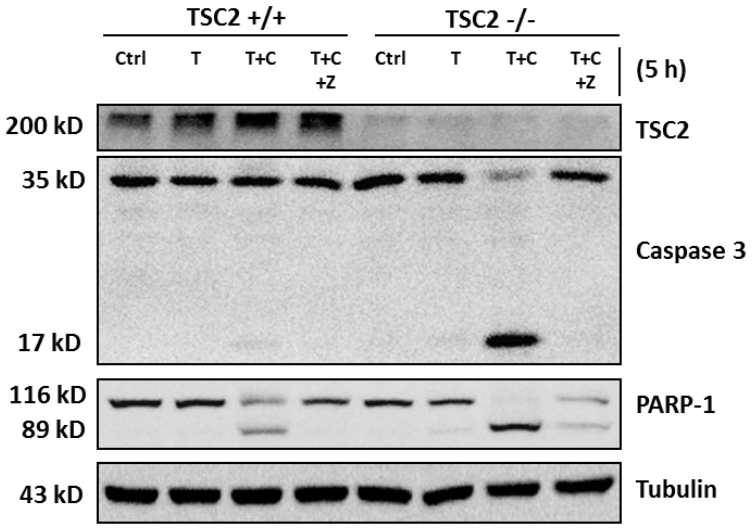


Figure 3.3 $TSC2^{-/-}$ MEFs are more sensitive to cell death induced by oxidative stress and TNFα stimuli. (A) Effects of hydrogen peroxide (H_2O_2) treatment on cell death in TSC2 MEFs (left panel). Cells were treated with H_2O_2 (0.5 mM) for 16 hours. Effects of nitric oxide on cell death in TSC2 cells (right panel). Cells were treated with sodium nitroprusside (SNP, 0.5 mM) for the indicated time. (B, next page) Effects of TNFα on cell death in the cells. TSC2 MEFs were treated with TNFα (T, 10 ng/ml), with or without cycloheximide (C, 10 μM) and zVAD (Z, 20 μM) for 5 hours. Cell lysates were analysed by western blotting using antibodies specific to caspase 3 and PARP-1 cleavages. Tubulin served as loading control.

B



3.2.2 Impaired basal and inducible autophagy in TSC2$^{-/-}$ cells due to constitutive mTORC1 activation

The loss of either TSC1 or TSC2 will lead to constitutive activation of mTORC1 (Garami et al., 2003). Since mTORC1 negatively regulates autophagy (Jung et al., 2009), we postulate that hyperactivation of mTORC1 could thus limit autophagy in TSC2$^{-/-}$ cells. Here we first compared the basal autophagy level in TSC2$^{+/+}$and TSC2$^{-/-}$ cells. As shown in Figure 3.4, there was high level of S6K (T389) phosphorylation in TSC2$^{-/-}$ cells, indicating higher activity of mTORC1. Interestingly, LC3-II is found to be lower and p62 level is higher in untreated TSC2$^{-/-}$ cells comparing to TSC2$^{+/+}$cells (Figure 3.4), suggesting the lower level of basal autophagy in TSC2$^{-/-}$ cells. Next, we tested the autophagy level induced by starvation. We found that the p-S6K is completely diminished in TSC2$^{+/+}$cells but not in TSC2$^{-/-}$ cells when cultured with EBSS within the first 2 hours, suggesting the suppression of mTORC1 activity by EBSS in TSC2$^{+/+}$cells, while the mTORC1 activity is not significantly affected in TSC2$^{-/-}$ cells. In order to assess the level of autophagy flux, we used chloroquine diphosphate (CQ), which is able to inhibit

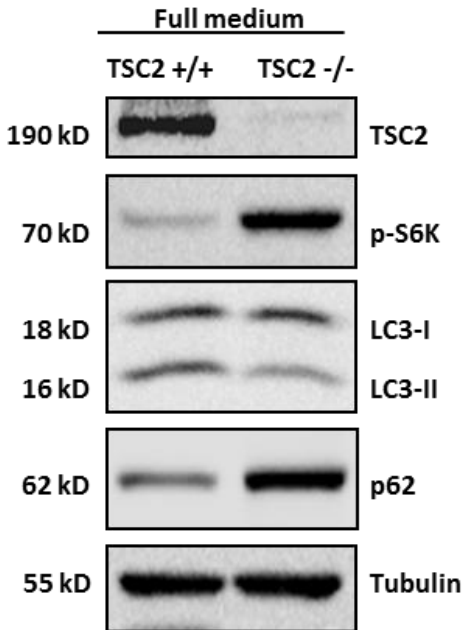


Figure 3.4 Basal autophagy level is lower in TSC2$^{-/-}$ cells. Cells were collected after incubated in fresh medium overnight without any treatment. Cell lysates were analysed with western blotting using indicated antibodies.

autophagosome maturation and degradation via neutralizing the lysosomal pH (Mizushima et al., 2010; Shen and Codogno, 2011). Interestingly, the addition of CQ alone increased LC3-II level without affecting p-S6K, which suggests that the effect of CQ is independent of mTORC1 activity. Notably, EBSS induced significant increase of LC3-II, especially in the presence of CQ in TSC2$^{+/+}$cells. In contrast, the changes of LC3-II in TSC2$^{-/-}$ MEFs are much less evident, suggesting a lower level of autophagy induced by EBSS in TSC2$^{-/-}$ cells (Figure 3.5A). Consistently, there was evident reduction of p62 protein level in wild type cells, but not in TSC2$^{-/-}$ cells (Figure 3.5A). Similar patterns of both LC3-II and p62 were also observed in cells with prolonged incubation with EBSS (for up to 24 hours) (Figure

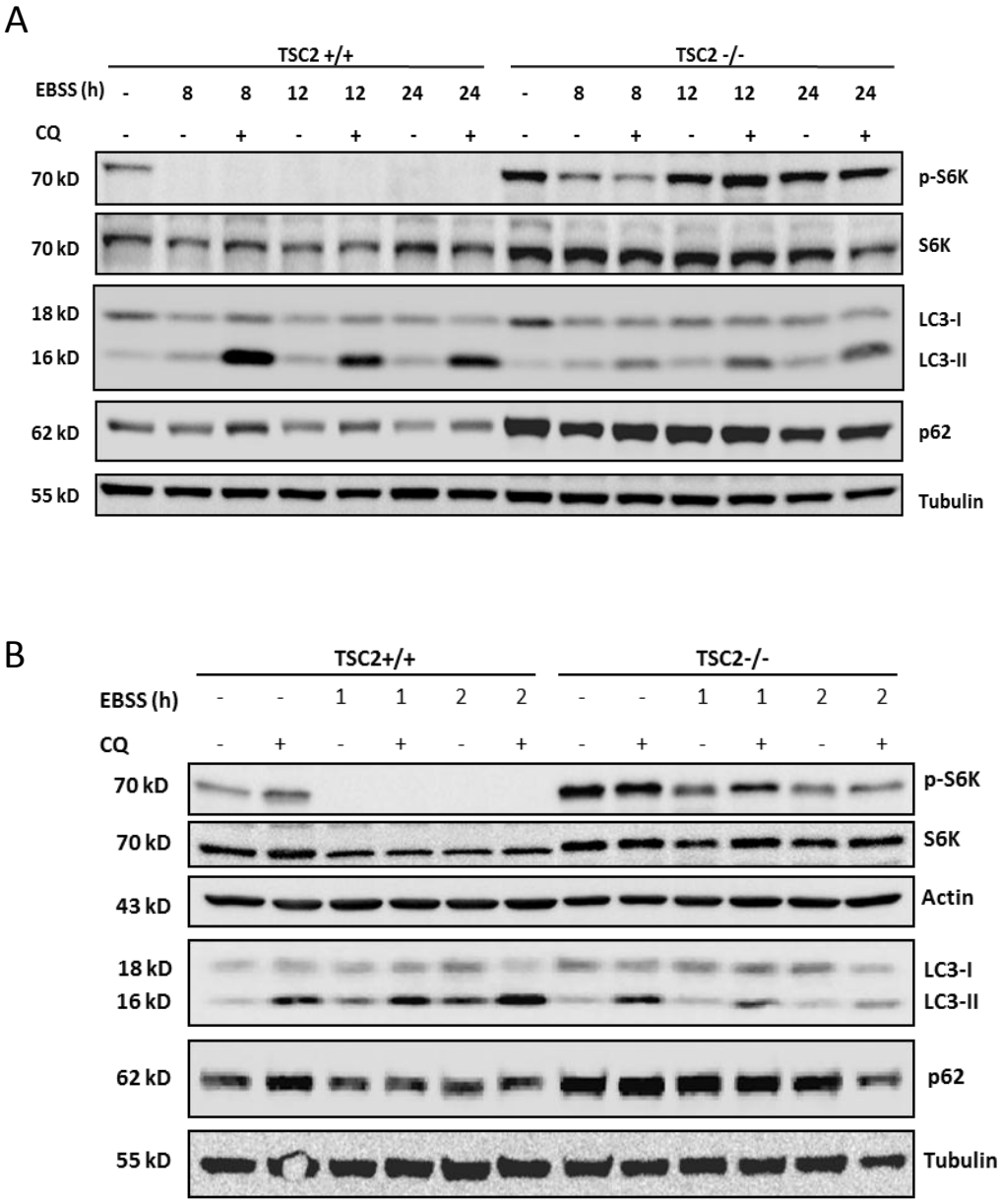


Figure 3.5 Lower inducible autophagy levels in TSC2$^{-/-}$ cells. (A) and (B) Induction of autophagy in TSC2 cells using EBSS. Cells were treated with EBSS in the presence or absence of chloroquine diphosphate (CQ, 10 μM) for the indicated period of time. Cell lysates were prepared for western blot for detection of various indicated markers. Actin and tubulin served as loading controls for the cell lysates.

3.5B). Interestingly, in TSC2$^{-/-}$ cells, starvation led to reduction of the p-S6K level in TSC2$^{-/-}$ cells within the first 8 hours and then was restored from 12 hours onwards (Figure 3.5A and 3.5B). Taken together, these data suggest that the basal

and inducible autophagy in TSC2$^{-/-}$ is impaired, possibly due to the constitutive activation of mTORC1.

3.2.3 Suppression of autophagy sensitizes EBSS-induced cell death in TSC2$^{+/+}$, but not in TSC2$^{-/-}$ cells

Here we used two experimental approaches to test if autophagy inhibition would have any impact on the cell death induced by EBSS in both type of cells. In our first approach, we used CQ to inhibit autophagy induced by starvation. As shown in Figure 3.6, the addition of CQ alone into fed cells did not induce any cell death. Notably, the addition of CQ was able to enhance cell death in cells treated under EBSS starvation. Interestingly, the death enhancing effect of CQ was much more evident in TSC2$^{+/+}$as compared to TSC2$^{-/-}$ cells. The percentage of cell death with CQ addition in starved TSC2$^{+/+}$cells increased from 38.50% to 50.34%, as compared to the slight increase from 58.13% to 64.58% in starved-only TSC2$^{-/-}$ cells (Figure 3.6B). Thus, these results suggest that (i) autophagy plays a pro-survival role in MEFs under starvation conditions; and (ii) CQ is less effective in TSC2$^{-/-}$ cells due to the lower inducible autophagy level.

Another approach used here was to knockdown Atg7, a key Atg in autophagosome expansion and completion by conjugating Atg12 to Atg5 (Cecconi and Levine, 2008). As shown in Figure 3.7A, starvation led to a significant increase of LC3-II level in cells with control NS (non-specific) siRNA, while such changes were prevented in cells treated with Atg7 siRNA in TSC2$^{+/+}$ cells. As expected, the suppressive effect of Atg7 knockdown is much less evident in the TSC2$^{-/-}$ cells (Figure 3.7B). As shown in Figure 3.8B, Atg7 knockdown in

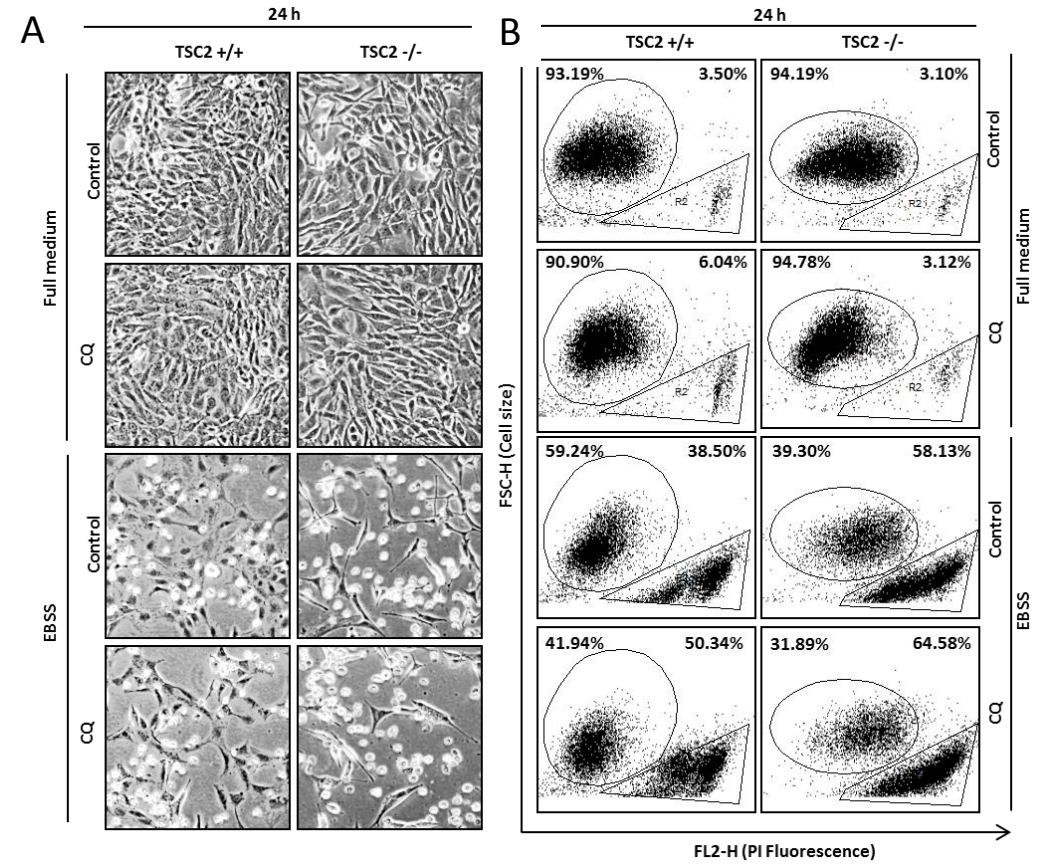


Figure 3.6 Autophagy inhibition with CQ sensitizes starved cells to death. (A) Morphological representative of cell death in TSC2 cells. Cells were treated in full medium or in EBSS, with or without CQ for 24 hours. CQ pretreatment was conducted in full medium for 2 hours and washed with 1X PBS prior to addition of full medium or EBSS in the presence or absence of CQ. Cells were examined and photographed with an inverted microscope (X 100). (B) PI live exclusion staining for cell death quantification. Cells were treated as in panel A and cell death was measured as described in Figure 3.1B and 3.2A. (C) Statistical significance (**p<0.01) of data from panel B was indicated in the bar chart.

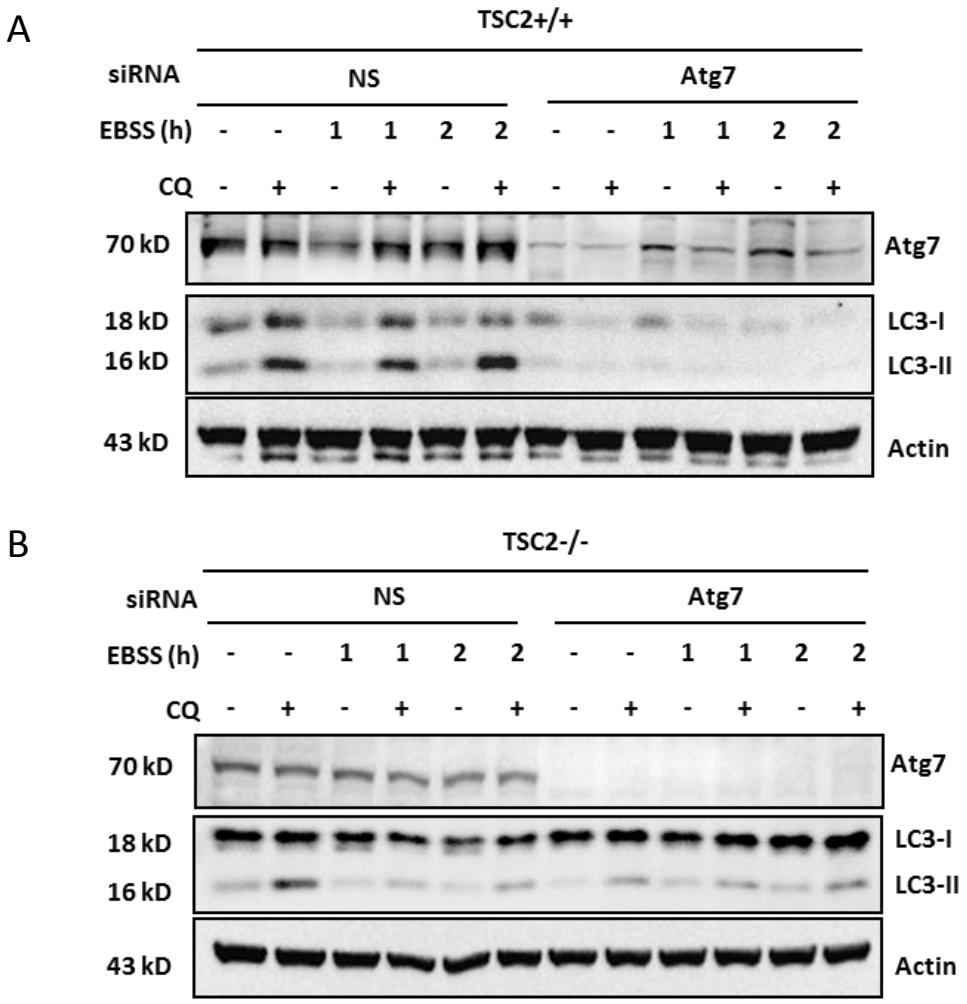
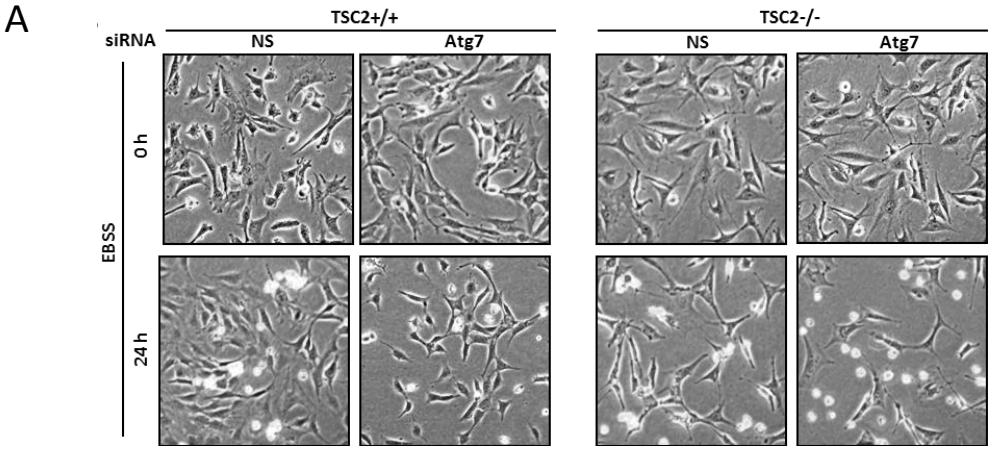


Figure 3.7 Atg7 knockdown inhibits autophagy. (A) and (B) Effects of Atg7 knockdown in TSC2 cells. After knockdown, $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were treated as described earlier in Figure 3.5. Cell lysates were subjected to western blotting for the indicated markers. Actin served as loading control.



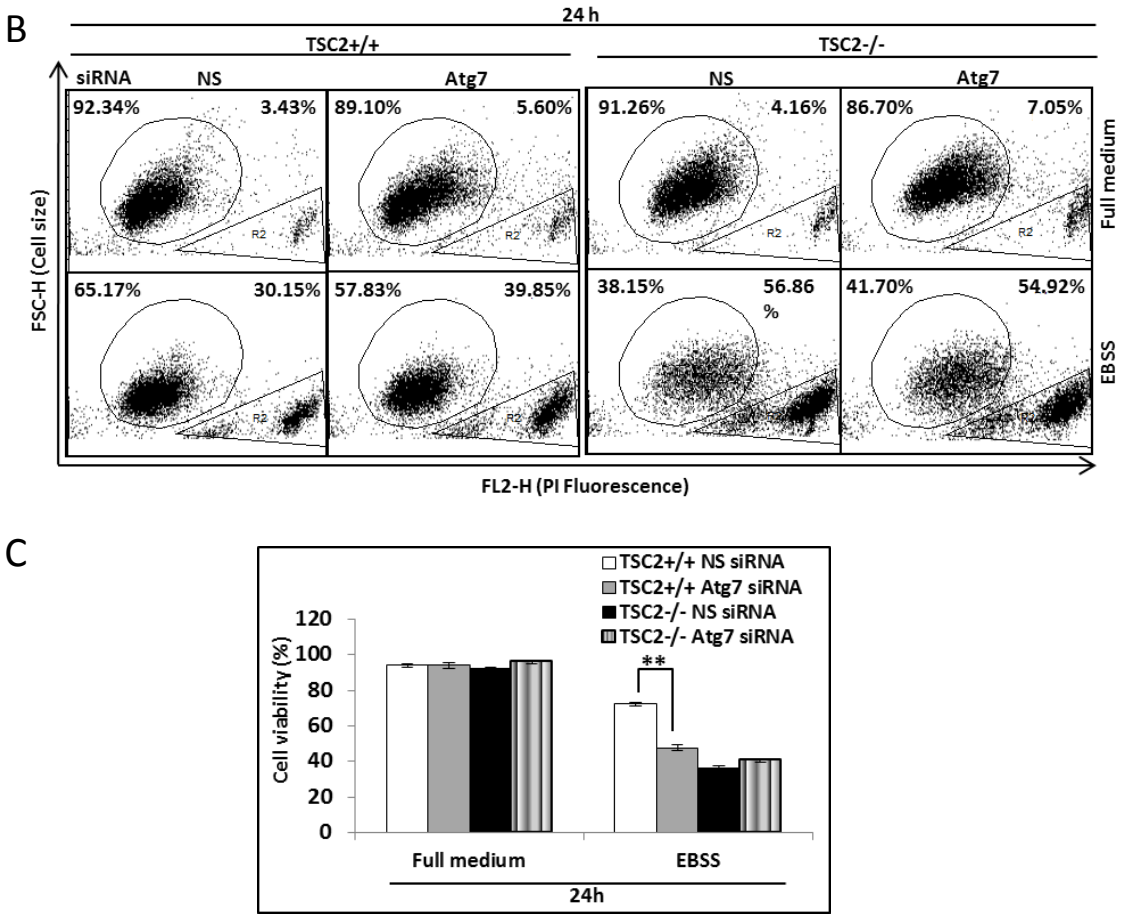


Figure 3.8 Autophagy inhibition with Atg7 knockdown sensitizes starved cells to death. Atg7 knockdown sensitizes TSC2$^{+/+}$cells to cell death but not in TSC2$^{-/-}$ cells. (A) Morphological representative of TSC2 cells. Cells were treated as described in Figure 3.7, examined and photographed with an inverted microscope (X 100). (B) PI live exclusion staining for cell death quantification. Cell death was measured as described earlier in Figure 3.1B and 3.2A. (C) Statistical significance ($^{**}p$<0.01, *t*-test) of respective treatments was indicated in bar chart.

EBSS-treated TSC2$^{+/+}$ cells increased the percentage of dead cells from 30.15% to 39.85%, whereas in starved TSC2$^{-/-}$ cells, the percentage of dead cells in starved TSC2$^{-/-}$ cells (56.86%) did not further increase upon Atg7 knockdown (54.92%). Consistently, the sensitization effect of Atg7 knockdown on cell death under starvation was much more significant in TSC2$^{+/+}$ cells in comparison to TSC2$^{-/-}$ cells (Figure 3.8).

3.2.4 Activation of autophagy protects against EBSS-induced cell death in $TSC2^{-/-}$ cells

Since the suppression of autophagy is able to sensitize the $TSC2^{+/+}$ cells to EBSS-induced cell death, suggesting a pro-survival function of autophagy under starvation, we next tested whether autophagy induction would increase cell survival of $TSC2^{-/-}$ cells. We combined two approaches for autophagy activation in $TSC2^{-/-}$ cells: treatment with rapamycin, a specific mTORC1 inhibitor, and raptor knockdown. First, we observed that rapamycin treatment or raptor knockdown in $TSC2^{-/-}$ cells effectively suppressed mTOR activity, as evidenced by significant suppression of S6K phosphorylation (Figure 3.9). Interestingly, the 4EBP1 (T37/46) phosphorylation (p-4EBP1) was found to be more resistant to rapamycin or raptor knockdown alone, while combination of both treatments led to a significant reduction of p-4EBP1 level (Figure 3.9). Consistently, either rapamycin or raptor knockdown was able to induce autophagy in $TSC2^{-/-}$ cells cultured in either full medium or EBSS, based on increased LC3-II protein level with the addition of CQ, as observed in lane 3 and comparing lane 8 to lane 9. Although the addition of rapamycin into cells with raptor knockdown does not further enhance the autophagic flux (comparing lane 11 and 13), we did observe higher level of autophagic flux (LC3-II level) with combined treatment of rapamycin and raptor knockdown (lane 13) when comparing to rapamycin only (lane 6).

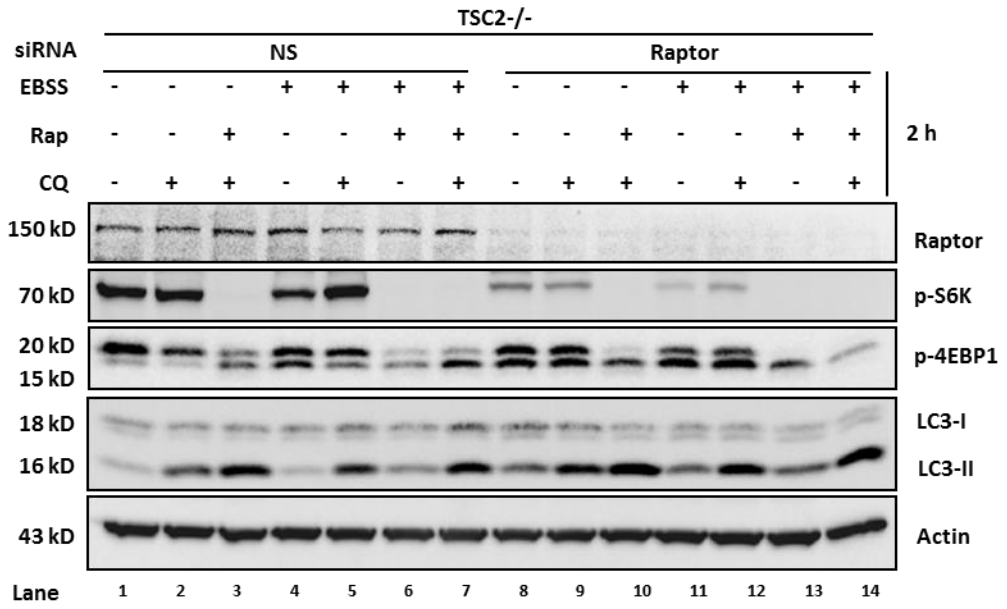
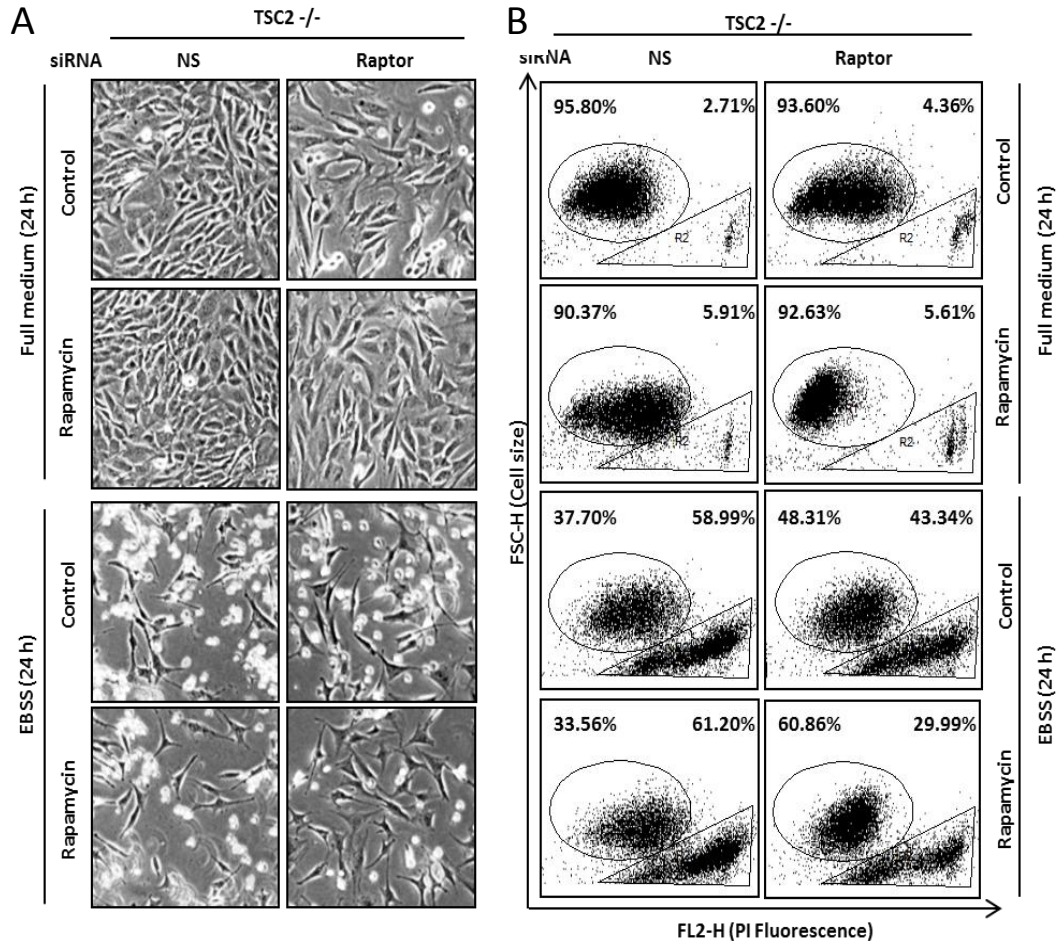


Figure 3.9 Raptor siRNA knockdown in TSC2$^{-/-}$ cells increases autophagy. TSC2$^{-/-}$ cells with control NS (non-specific) or raptor knockdowns were treated with full medium or EBSS in the presence or absence of CQ (10 μM) and rapamycin (10 nM) for the indicated time. Cell lysates were collected and subjected to western blots for the indicated markers.

Next, we examined cell death under the conditions described above. As shown in Figure 3.10B, rapamycin treatment alone failed to reduce the percentage of cell death induced by EBSS (percentage of cell death was 58.99% in starved TSC2$^{-/-}$ as compared to 61.20% with rapamycin addition), whereas raptor knockdown offered moderate protection against EBSS-induced cell death (43.34%). Consistent with the degree of mTOR suppression and autophagy induction as shown in Figure 3.9, the combination of both rapamycin and raptor knockdown well protected TSC2$^{-/-}$ MEFs against EBSS-induced cell death (percentage of cell death reduced from 61.2% to 29.99%). Such observations thus further support the notion that autophagy is an important pro-survival mechanism in response to starvation.



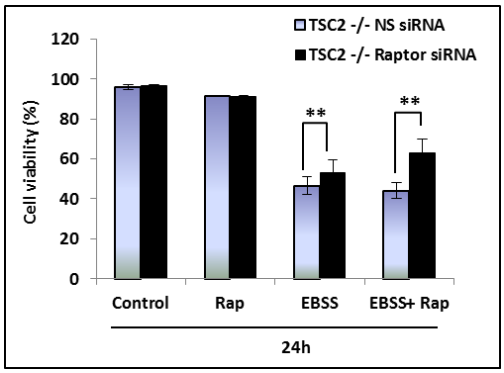


Figure 3.10 Autophagy induction through raptor knockdown rescues cell death in TSC2$^{-/-}$ cells. (A) Morphological representatives of TSC2$^{-/-}$ cells treated with full medium or EBSS, with or without rapamycin for 24 hours. Cells were examined and photographed under an inverted microscope (X 100). (B) PI live exclusion staining for cell death quantification. Cell death was measured as described earlier. (C) Statistical significance ($^{**}p$<0.01, *t*-test) of respective treatments in (B) was indicated in the bar chart.

3.2.5 Nutrients supplementation protects against cell death in $TSC2^{+/+}$cells, but enhances cell death in $TSC2^{-/-}$ cells under starvation condition

Here, in order to further establish the pro-survival function of autophagy in $TSC2^{-/-}$ cells, we investigated whether nutrients supplementation will affect autophagy and cell death. Insulin-like growth factor-1 (IGF-1) together with leucine, an essential amino acid, is known to activate mTORC1 and suppress autophagy, via activation of the PI3K-Akt-TSC pathway (Meijer and Codogno, 2009). In this study, the addition of IGF-1+leucine activates PI3K-Akt-mTORC1 pathway, as seen by the phosphorylations of Akt (S473) and S6K (both shorter and longer exposures) in the $TSC2^{+/+}$ MEFs (Figure 3.11). The absence of Akt phosphorylation (p-Akt) in $TSC2^{-/-}$ MEFs in response to IGF-1+leucine is believed to be due to the negative feedback loop between activated S6K on IGF receptor signaling (Huang et al., 2009a). Similar to the earlier observations, EBSS starvation caused slight reduction of p-S6K level in $TSC2^{-/-}$ cells, while the addition of IGF-1+leucine restored its original level.

The supplementation of nutrients also affects autophagy induced by EBSS in both type of cells. As shown in Figure 3.11, the addition of IGF-1+leucine significantly reduced EBSS-induced increase of LC3-II in $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs, although in the level of autophagy in $TSC2^{-/-}$ MEFs was much lower, being consistent with earlier observations in Figure 3.5. Intriguingly, the addition of IGF-1+leucine had opposite effects on EBSS-induced cell death in these two cell types: it almost completely abolished caspase 3 cleavage in $TSC2^{+/+}$ MEFs, while enhanced caspase 3 cleavage in $TSC2^{-/-}$ MEFs at eighth hour of treatment

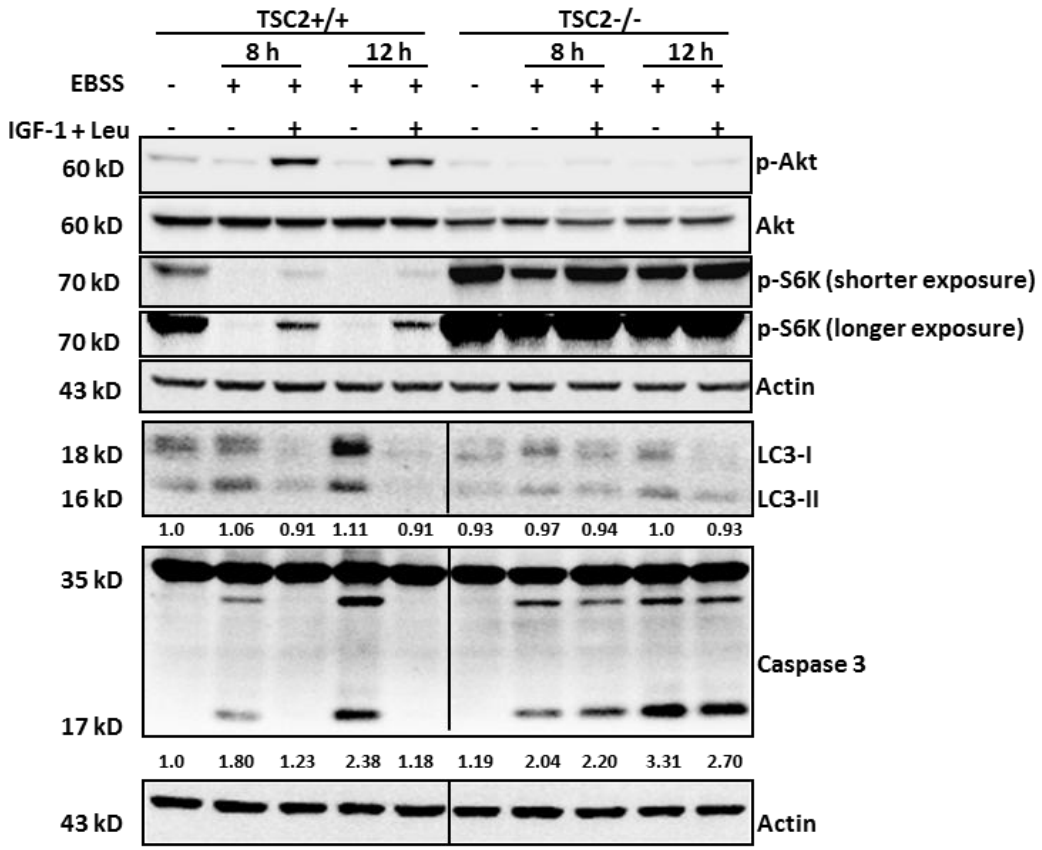


Figure 3.11 Effect of IGF-1+Leu (insulin growth factor-1 and leucine) on mTORC1 activation, autophagy and cell death in TSC2$^{-/-}$ cells. TSC2$^{-/-}$ cells were starved in EBSS for two hours, followed by the addition of IGF-1 (200 nM) + Leu (0.2 mg/ml) for the indicated time. Cell lysates were subjected to western blotting for the markers. Respective lane intensity of LC3-II and caspase 3 cleavage were quantified using Kodak Imaging Software as fold change to control.

(lane 8, Figure 3.11). Similar results were observed when cell death was determined by cell morphological changes (Figure 3.12A) and PI live exclusion staining (Figure 3.12B). Therefore, data from this part of our study further support the notion that autophagy plays a vital function in maintaining cell survival under starvation conditions, especially in TSC2$^{-/-}$ cells.

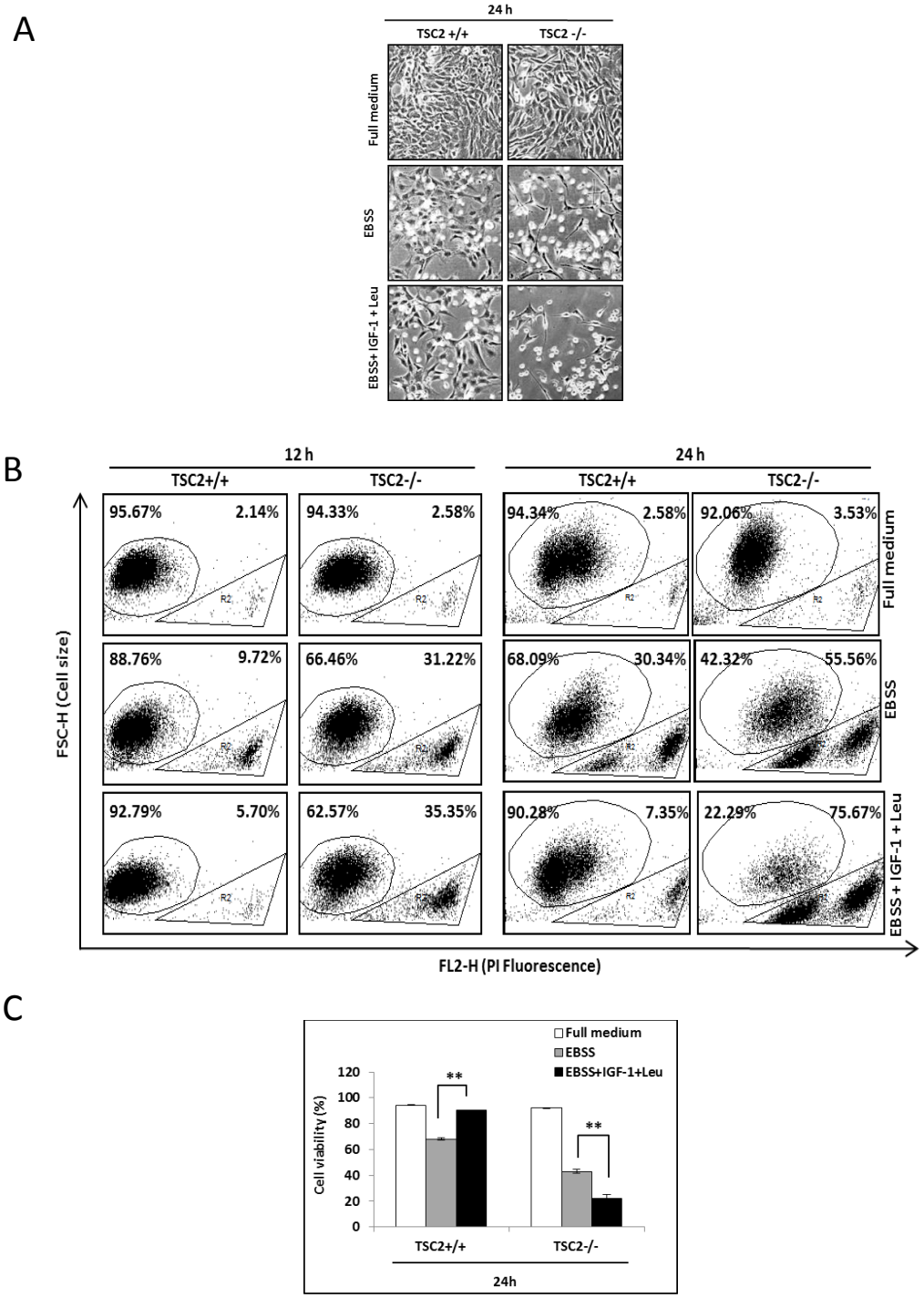


Figure 3.12 Nutrients supplementation further sensitizes TSC2$^{-/-}$ cells to cell death. (A) Morphological representatives of cells treated as in Figure 3.11 for the indicated time. Cells were examined and photographed under an inverted microscope (X 100). (B) PI live exclusion assay for cell death quantification. Cell death was quantified using PI as described earlier. (C) Statistical significance ($^{**}p$<0.01, *t*-test) of respective treatments in panel B was indicated in the bar chart.

3.3 Discussion

It has been well established that the dysfunction of TSC leads to constitutive activation of mTORC1, a process implicated in various pathological conditions including cancer (Inoki and Guan, 2009). On the other hand, the TSC-mTOR signaling axis is also known to be a key negative regulator of autophagy, directly upstream of the Atg1/ULK complex (He and Klionsky, 2009). Cells with TSC deficiency have been found to be hypersensitive to various cell death stimuli, such as TNFα (Ghosh et al., 2006), glucose deprivation (Lee et al., 2007a) and ER stress (Di Nardo et al., 2009; Kang et al., 2011). At present, it has not been systematically studied whether autophagy is involved in the hypersensitive nature of the TSC-deficient cells to cell death. In this study, we provide evidence suggesting that the constitutive activation of mTORC1 impaired autophagy (both basal and inducible), which then contributes to the hypersensitivity of the $TSC2^{-/-}$ cells to stress-induced apoptosis. Our findings thus shed new lights onto the intricate relationship among the TSC-mTOR signaling pathway, autophagy and cell death.

mTORC1 activation is known to suppress autophagy via targeting the ULK1-Atg13-FIP200 complex (He and Klionsky, 2009; Hosokawa et al., 2009; Jung et al., 2009). Therefore, the constitutive activation of mTORC1 in $TSC2^{-/-}$ cells is expected to lead to suppression of autophagy. In this study, we found that the basal level of autophagy in $TSC2^{-/-}$ was lower than the wild type cells, shown by the lower LC3-II and higher p62 protein level (Figure 3.4). More importantly, the inducible autophagy by starvation is also significantly impaired in $TSC2^{-/-}$

cells in comparison to the wild-type cells (Figure 3.5). It has been reported that mTORC1 activity in $TSC2^{-/-}$ cells is rather refractory to starvation (Smith et al., 2005). Consistently, we only observed partial reduction of p-S6K level in $TSC2^{-/-}$ cells after EBSS exposure (Figure 3.5A) and the p-S6K level was largely restored to the basal level with prolonged EBSS treatment (for 12 hours, Figure 3.5B). In contrast, culturing in EBSS completely abolished p-S6K in $TSC2^{+/+}$ cells (Figure 3.5). Consistently, starvation (in EBSS) is more effective for the induction of autophagy in $TSC2^{+/+}$ cells (Figure 3.5). Moreover, rapamycin or raptor knockdown was able to enhance autophagic flux in $TSC2^{-/-}$ cells (Figure 3.9), suggesting that the basic autophagy machinery is not defective in $TSC2^{-/-}$ cells. Therefore, it is believed that it is the constitutive activation of mTORC1 that leads to autophagy suppression in $TSC2^{-/-}$ cells.

In this study, we examined the function of autophagy in cell death under starvation via several approaches. On one hand, suppression autophagy by CQ or Atg7 knockdown was able to enhance cell death, which is much more effective in $TSC2^{+/+}$ cells than the $TSC2^{-/-}$ cells (Figure 3.6 and 3.8). Such data could be understood as the autophagy level was already low in $TSC2^{-/-}$ and further suppression will only give rise to less significant effect. On the other hand, activation of autophagy by rapamycin and raptor knockdown was effective in protecting starvation-induced cell death in $TSC2^{-/-}$ cells (Figure 3.10). Rapamycin specifically inhibits mTORC1 via the formation of immunophilin FKBP12-rapamycin complex that allosterically interrupts the FRB (FKBP12-rapamycin binding) domain of mTOR (Choi et al., 1996). It is also known that some of the

mTOR activity is insensitive to rapamycin, such as the phosphorylation of 4EBP1 at T37 and T46 sites (Gingras et al., 1999; Wang et al., 2005).

It is interesting to note that combination of raptor knockdown and rapamycin could offer more effective protection against starvation-induced cell death than rapamycin or raptor knockdown alone (Figure 3.10). Such findings are generally consistent with a very recent report on ER stress-mediated apoptosis in $TSC2^{-/-}$ cells (Kang et al., 2011). In their study, rapamycin effectively blocked apoptosis in glucose-deprived $TSC2^{-/-}$ cells, but not in ER-stress induced cells. Similarly, raptor knockdown rescues ER-stress induced cell death (Kang et al., 2011). One possible explanation is that there are rapamycin-insensitive functions of mTOR which may mediate the resistance to cell death. In fact, rapamycin was able to completely abolish p-S6K, while only partly reduce p-4EBP1 (Figure 3.9). Similar trend in p-4EBP1 was found for the effect of raptor knockdown, while the combination of these two approaches led to more evident suppression of p-4EBP1 (Figure 3.9). It is thus believed that either rapamycin or raptor knockdown alone are insufficient to block all mTORC1 functions. Moreover, since the induction of autophagy by the combination of rapamycin and raptor knockdown was not proportional to the blockage of mTORC1 activity and protection against cell death, it is possible that not all mTOR-related functions on cell death are mediated via autophagy.

One interesting observation in this study is that EBSS is able to induce cell death, while there is no obvious cell death induced by rapamycin or raptor knockdown, although all these treatments can block the mTORC1 function. There

are several possible explanations. First, only very low concentration of rapamycin (10 nM) was used in this study. It is well known that higher concentration of rapamycin is capable of inducing cell death in certain cancer cell lines (Guertin and Sabatini, 2005). Second, it has been reported that EBSS is able to induce cell death (apoptosis) via multiple mechanisms, such as oxidative stress (Scherz-Shouval et al., 2007). Third, Akt is no longer activated in EBSS-treated cells (Figure 3.11). As Akt activation plays an important role in maintaining cell survival and preventing apoptosis (Franke et al., 2003), it is possible that the different Akt activation status contributes differently to cell death/survival under different treatments as described above.

Another intriguing observation from this study is that nutrient supplementation has opposite effect on $TSC2^{+/+}$and $TSC2^{-/-}$ cells: In EBSS-starved cells, addition of IGF-1+leucine was able to reduce cell death in $TSC2^{+/+}$cells, but aggravate cell death in $TSC2^{-/-}$ cells (Figure 3.11 and 3.12). An earlier study has shown that starvation is able to elicit a *p53*-dependent apoptosis pathway in cells with TSC deficiency and hyperactivation of mTORC1 (Lee et al., 2007a). Since the TSC2 cells used in our study are *p53* deficient (Zhang et al., 2003a), the apoptotic pathway is likely to be *p53*-independent. In $TSC2^{+/+}$ cells, supplementation of IGF-1+leucine leads to evident activation of Akt, a well-established pro-survival molecule to block apoptosis via multiple pathways (Franke et al., 2003). Theoretically, nutrient supplementation (IGF-1+leucine) is able to elicit two pathways that are able to affect the cell fate: (i) activation of the Akt for cell survival (Franke et al., 2003), and (ii) suppression of autophagy for

cell death (He and Klionsky, 2009).

In this study, nutrient supplementation protects $TSC2^{+/+}$ cells, but enhances $TSC2^{-/-}$ cells to EBSS-induced apoptosis. Therefore, it is believed that in $TSC2^{+/+}$ cells, the Akt activation induced by nutrient supplementation plays a more dominant role in protecting against cell death. Conversely, in $TSC2^{-/-}$ cells, nutrient supplementation fails to activate Akt due to the negative feedback from activated S6K on IRS-1, an essential component of insulin receptor (Harrington et al., 2004). Rather it was able to further enhance mTORC1 activation and reduce autophagy, leading to increased susceptibility of $TSC2^{-/-}$ cells to starvation-induced cell death. Such findings are generally consistent with our previous report in which the activation of PI3K-Akt-mTOR pathway suppresses autophagy and promotes cell death via necrosis (Wu et al., 2009).

AMPK is another critical mediator upstream of mTOR in modulation of autophagy (Meijer and Codogno, 2007; Papandreou et al., 2008). In this study, we did not study the role of AMPK as it was widely believed that involvement of AMPK in autophagy was mainly via its effect on TSC2 to suppress mTOR (Gwinn et al., 2008; Inoki et al., 2003b). However, several very recent reports have discovered the new functions of AMPK on autophagy, including its direct effect on the ULK1 complex (Egan et al., 2011; Kim et al., 2011). Therefore, it would be important to investigate the involvement of AMPK in the suppressed autophagy level in $TSC2^{-/-}$ cells in future.

Despite the general belief that autophagy is a pro-survival mechanism under starvation condition (Yang and Klionsky, 2010a), at present whether

autophagy is involved in the hypersensitivity of $TSC2^{-/-}$ cells to cell death stimuli is not clear. An earlier report examined the role of autophagy in cell death induced by glucose deprivation in $TSC2^{-/-}$ cells (Choo et al., 2010). In their study, rapamycin was found to be highly effective in protecting cell death induced by glucose deprivation. Although rapamycin is able to induce autophagy in $TSC2^{-/-}$ cells, suppression of such autophagy failed to affect the protective effect of rapamycin, thus it is concluded that autophagy is not involved in the protective effect of rapamycin (Choo et al., 2010). Unfortunately, the authors did not examine and compare autophagy induction by glucose deprivation in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells, and whether such autophagy contributes to the different susceptibility of both cells to cell death under glucose deprivation. Another possible reason for such discrepancies is the different nature of starvation used. In our study, EBSS contains a considerable amount of glucose (1 g/L), while it is completely deprived of amino acids and growth factors in their study. It remains to be further investigated whether these two forms of starvation engage autophagy's pro-survival function differently in $TSC2^{-/-}$ cells.

Taken together, data from our study provide the first evidence showing the importance of autophagy in mediating the survival of cells with deficiency of TSC function. As shown in Figure 3.13, the constitutive activation of mTOR leads to suppression of autophagy, which serves as an important pro-survival mechanism in response to starvation. Suppression of autophagy by CQ or Atg7 knockdown enhances the cells to starvation-induced apoptosis while rapamycin and raptor knockdown would protect cells via suppression of mTOR activation and

subsequent induction of autophagy. Such findings shed new lights onto the intricate relationship among the TSC-mTOR signaling pathway, autophagy and cell death. More importantly, our results may provide clues for developing autophagy-targeted intervention strategies for the treatment of TSC-related pathologies.

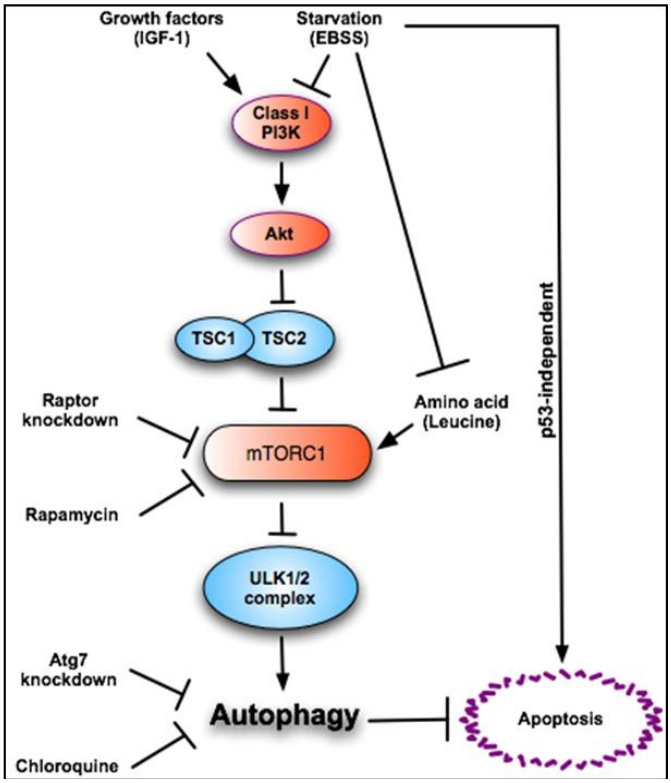


Figure 3.13 The involvement of TSC in the regulation of autophagy and cell death. In the absence of TSC, constitutive mTORC1 activation suppresses autophagy and thus contributes to the susceptibility of TSC-deficient cells to cell death.

CHAPTER 4

TSC PROTEIN PROMOTES OXIDATIVE STRESS-MEDIATED JNK ACTIVATION VIA DISRUPTION OF MKP-1 FUNCTION

4.1 Introduction

Oxidative stress is defined as the elevated production of reactive oxygen species (ROS), due to insufficient antioxidant mechanism for the defense against the production of ROS during cellular processes (Halliwell and Cross, 1994; Sies, 1997). At present, oxidative stress has been extensively studied and it is known to be closely implicated in human diseases such as neurodegeneration, cancer, autoimmune diseases and ageing, through damage to DNA, lipids and proteins (Weinberg and Chandel, 2009). Hydrogen peroxide (H_2O_2) is a common ROS produced in cells as a byproduct of oxidative metabolism. Despite the presence of antioxidant enzymes and other non-enzymatic antioxidants that rapidly detoxify ROS in cells, a perturbed cellular aerobic metabolism may lead to ROS accumulation, and subsequently causes cellular injury (Weinberg and Chandel, 2009). On the other hand, ROS are known to be important signaling molecules and mediate several signaling cascades that are closely involved in numerous physiological and pathological processes (D'Autreaux and Toledano, 2007; Gamaley and Klyubin, 1999; Thannickal and Fanburg, 2000).

Among them, the c-Jun-N-terminal kinase (JNK) signaling is one of the key pathways elicited by ROS (Shen and Liu, 2006; Torres, 2003). JNK has been extensively studied and its activation is mediated through the three-tiered signaling modules, initiated from the upstream mitogen-activated protein kinase kinase kinase (also known as MAPKKK, MKKK or MAP3K), to MAPKK (MAP2K or MKK) and subsequently to MAPK (Marshall, 1994). At least 14 MAP3Ks have been found to activate JNK, including apoptosis signal-regulating

kinase 1 (ASK1), while the direct upstream of JNK are MKK4 and MKK7 (Seki et al., 2012). Meanwhile, JNKs are dephosphorylated by MAPK phosphatases (MKPs), which are dual-specificity phosphatases (Keyse, 2008). The most effective substrate of JNK is c-Jun, which is a member of the nuclear transcriptional factor AP-1, together with other JNK substrates such as JunB, JunD, and Fos (Karin and Gallagher, 2005).

At present there is evidence suggesting the crosstalks between the JNK and mTOR signaling pathways. It has been previously reported that JNK is required for the feedback inhibition of the insulin signaling cascade of IRS-1 (Aguirre et al., 2000; Hilder et al., 2003; Lee et al., 2003), which lies upstream of the PI3K-Akt-mTOR pathway (Cohen, 2006). The absence of JNK1 resulted in significant improvements of insulin sensitivity as well as enhancement in insulin receptor signaling capacity in obese mice models (Hirosumi et al., 2002). JNK is also responsible for raptor phosphorylation to stimulate mTORC1 in intestinal tumour cells (Fujishita et al., 2011) as well as during osmotic stress (Kwak et al., 2012). However, up to date little is known whether the TSC-mTORC1 pathway has any functional effect on the JNK signaling pathway mediated by various stress factors.

Despite numerous studies have indicated that TSC-null cells are more susceptible to various cell death inducers, the function of TSC in oxidative-stress response remains elusive. In this study, we first found that JNK activation was impaired in both TSC1- and TSC2- deficient cells. We also found that mTORC1 activity, autophagy and upstream MAP3K-MAP2K signaling were not involved

in the impaired JNK regulation observed in TSC-deficient cells. Interestingly, $TSC2^{-/-}$ cells have a significantly lower tyrosine phosphorylation with H_2O_2 treatment, and this possibly correlated with the significantly higher MKP-1 mRNA level observed in the $TSC2^{-/-}$ cells. Importantly, we found that TSC2 was co-immunoprecipitated with MKP-1, indicating a possible functional impact of TSC2 protein on MKP-1. Finally, we provided evidence suggesting that JNK impairment promoted necrotic cell death in $TSC2^{-/-}$ cells. We thus propose a novel role of TSC in regulating oxidative stress-mediated JNK signaling pathway via MKP-1.

4.2 Results

4.2.1 Activation of JNK is impaired in TSC-null MEFs

In order to test whether the TSC-mTORC1 signaling pathway has any functional impact on JNK activation under stress, we first explored H_2O_2-induced JNK activation in TSC2-null ($TSC2^{-/-}$) and TSC1-null ($TSC1^{-/-}$) MEFs. Interestingly, dose- and time-dependent treatments of H_2O_2 showed abrogated JNK activation in $TSC2^{-/-}$ (Figure 4.1A and 4.1B, respectively) as compared to its corresponding wild type ($TSC2^{+/+}$) cells. Moreover, there was reduced p-c-Jun, a downstream substrate of JNK (Seki et al., 2012). Since JNK signaling is also activated in response to other stress stimuli such as TNFα, ultraviolet (UV) radiation and nitric oxide (Karin and Gallagher, 2005), we asked if JNK signaling is also reduced with these stimuli in $TSC2^{-/-}$ cells. Consistently, we found that the JNK activation was much reduced in $TSC2^{-/-}$ cells stimulated with TNFα, UV and sodium nitroprusside (SNP), a nitric oxide donor (Figure 4.2A and 4.2B).

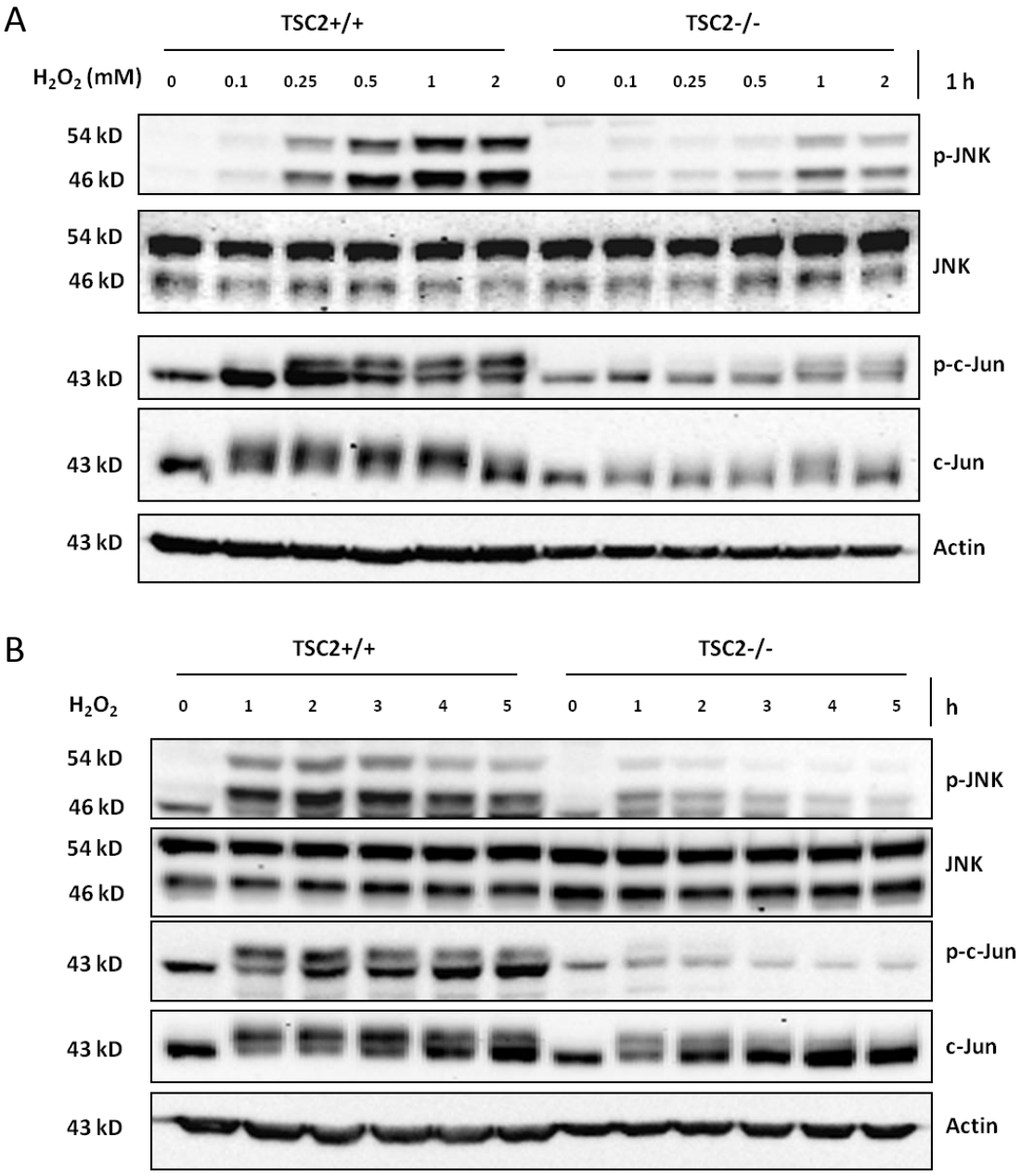


Figure 4.1 $TSC2^{-/-}$ cells are impaired in oxidative stress-induced JNK activation. (A) Dose-dependent effect of hydrogen peroxide (H_2O_2). $TSC2^{+/+}$ and $TSC2^{-/-}$ mouse embryonic fibroblasts (MEFs) were treated with different doses of H_2O_2 (0, 0.1, 0.25, 0.5, 1 and 2 mM, respectively) as indicated for 1 hour. (B) Time-dependent effect of H_2O_2. The TSC2 cells were treated with of H_2O_2 (0.5 mM) for the indicated period of time. Subsequently, cell lysates were collected and western blotting was performed for the indicated markers. Actin served as loading control.

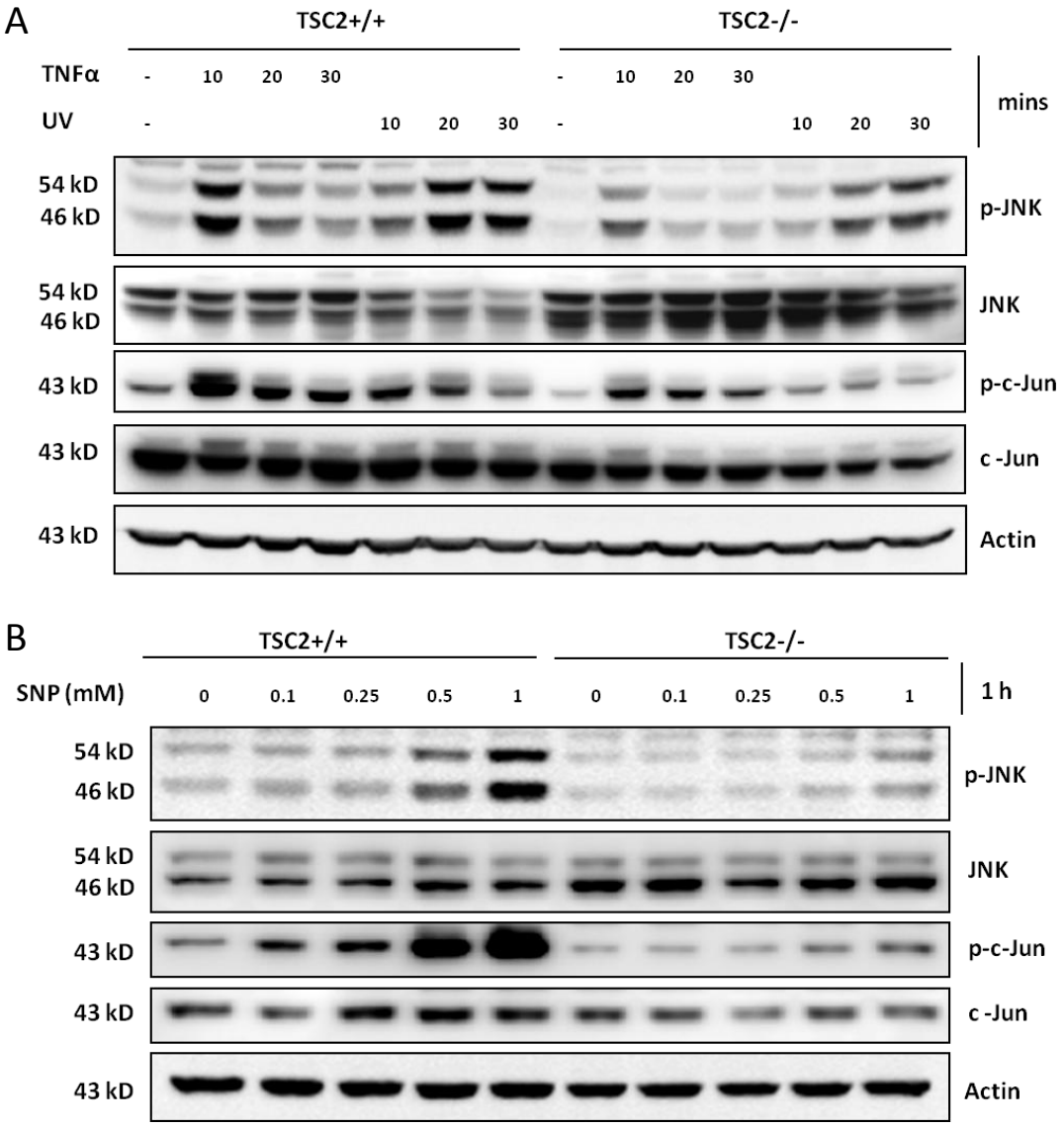


Figure 4.2 TSC2$^{-/-}$ MEFs have weakened JNK signaling activation induced by other stimuli. (A) Effect of pro-inflammatory cytokines and ultraviolet (UV) radiation in TSC2 cells. TSC2$^{+/+}$ and TSC2$^{-/-}$ MEFs were treated with TNFα (10 ng/ml) and UV for the indicated time. (B) Effect of nitric oxide in TSC2 cells. The nitric oxide donor, sodium nitroprusside (SNP) of different doses was used to stimulate the cells as indicated for 1 hour. Cell lysates were collected after designated treatments and western blotting was performed for the indicated markers. Actin served as loading control.

Similar results were also observed in TSC1$^{-/-}$ cells treated with H_2O_2 (Figure 4.3), TNFα (Figure 4.4A) and SNP (Figure 4.4B), respectively. Data from this part of

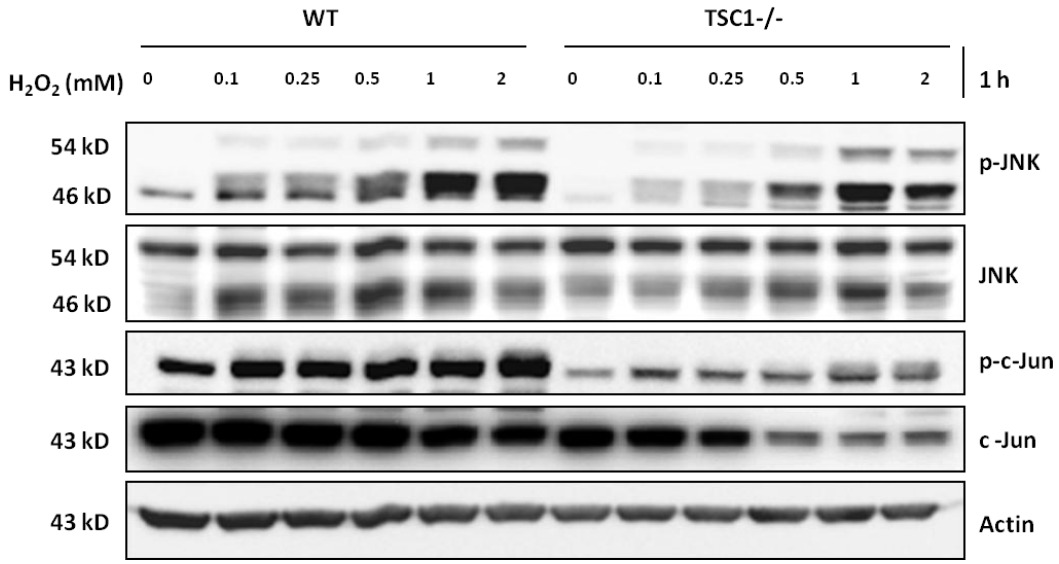


Figure 4.3 $TSC1^{-/-}$ MEFs have impaired JNK activation induced by H_2O_2. $TSC1^{+/+}$ (WT) and $TSC1^{-/-}$ MEFs were treated with different doses of H_2O_2 as indicated for 1 hour. After treatment, cell lysates were collected for western blotting. The indicated markers were blotted and actin served as loading control.

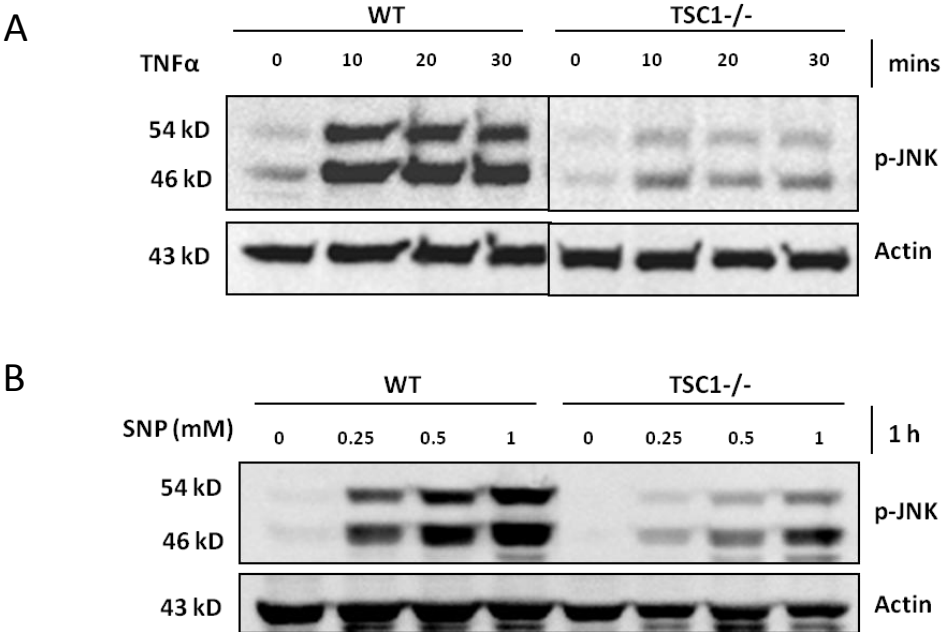


Figure 4.4 $TSC1^{-/-}$ MEFs have reduced JNK activation induced by other stimuli. (A) Effect of pro-inflammatory cytokines in TSC1 cells. The WT and $TSC1^{-/-}$ cells were treated with TNFα (10 ng/ ml) for the indicated time. (B) Effect of nitric oxide inducer in TSC1 cells. The MEFs were treated with different doses of SNP as described in Figure 4.2B. Cell lysates were collected. Subsequently, western blotting was performed for the indicated markers. Actin served as loading control.

our study thus demonstrate that deficiency of TSC protein impairs JNK activation in response to stress factors.

4.2.2 TSC2 protein is involved in the JNK signaling pathway

In order to confirm the role of TSC protein in JNK signaling, we then assessed the effects of TSC2 siRNA knockdown in $TSC2^{+/+}$ cells on H_2O_2–mediated JNK activation. As expected, TSC2 knockdown reduced JNK activation (Figure 4.5). To further determine the relevance of TSC2 toJNK signaling, we used the following three different approaches to reconstitute TSC2 expression in $TSC2^{-/-}$ MEFs. First, we transiently over-expressed TSC2 in $TSC2^{-/-}$ cells and then treated with H_2O_2. As shown in Figure 4.6, we found that the phosphorylation of JNK (p-JNK) is restored partially in transfected cells. The concurrent reduction of the p-S6K and p-4EBP1 level indicated the restoration of TSC complex function.

Second, we generated clones with stable reconstitution of TSC2 into $TSC2^{-/-}$ cells. As shown in Figure 4.7A, although the TSC2 level was rather low, we observed a reduced p-S6K, indicating the functionality of TSC2 in suppressing mTORC1 in $TSC2^{-/-}$ cells. Clones #4, #9, #10, and #11 were then further utilised to examine H_2O_2-induced JNK signaling. As shown in Figures 4.7B and 4.7C, respectively, clone #9 and #10 indeed showed significant restoration of p-JNK, indicating the role of TSC2 or TSC complex in the regulation H_2O_2-mediated JNK activation. Third, we took advantage of existing stable reconstituted MEFs (Huang et al., 2008), which were labeled as $TSC2^{-/-(+/+)}$ with pairing reconstituted Empty Vector, $TSC2^{-/-(EV)}$ cells as control (Figure 4.8A). The identity of the

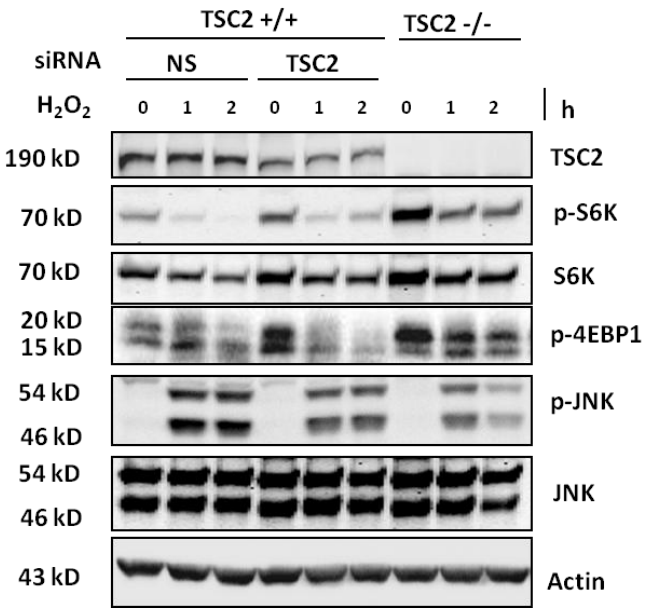


Figure 4.5 TSC2 knockdown impairs JNK phosphorylation. Non-specific (NS) siRNA that served as control or TSC2 siRNA knockdown in $TSC2^{+/+}$ cells was performed. After 48 hours, cells were treated with H_2O_2 (0.5 mM) for the indicated time. $TSC2^{-/-}$ cells were treated simultaneously. Cell lysates were subsequently collected for western blotting as described previously.

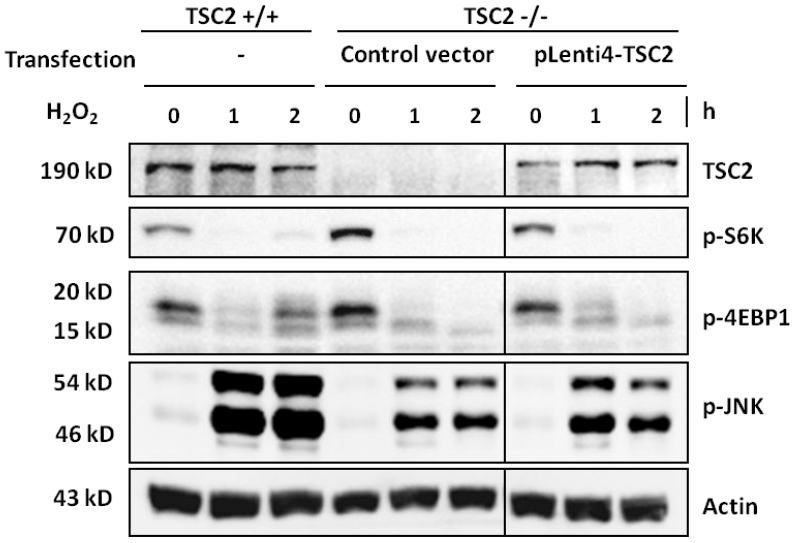


Figure 4.6 Overexpression of TSC2 restores JNK activation. $TSC2^{-/-}$ cells were transfected with a control vector or with the plasmid containing *TSC2* gene (pLenti4-TSC2). After 48 hours transfection, the cells (including $TSC2^{+/+}$ cells) were treated with 0.5 mM of H_2O_2 as the indicated time. The collected cell lysates were subsequently used for immunoblotting. Actin served as loading control. All the lanes were from the same western blot membrane.

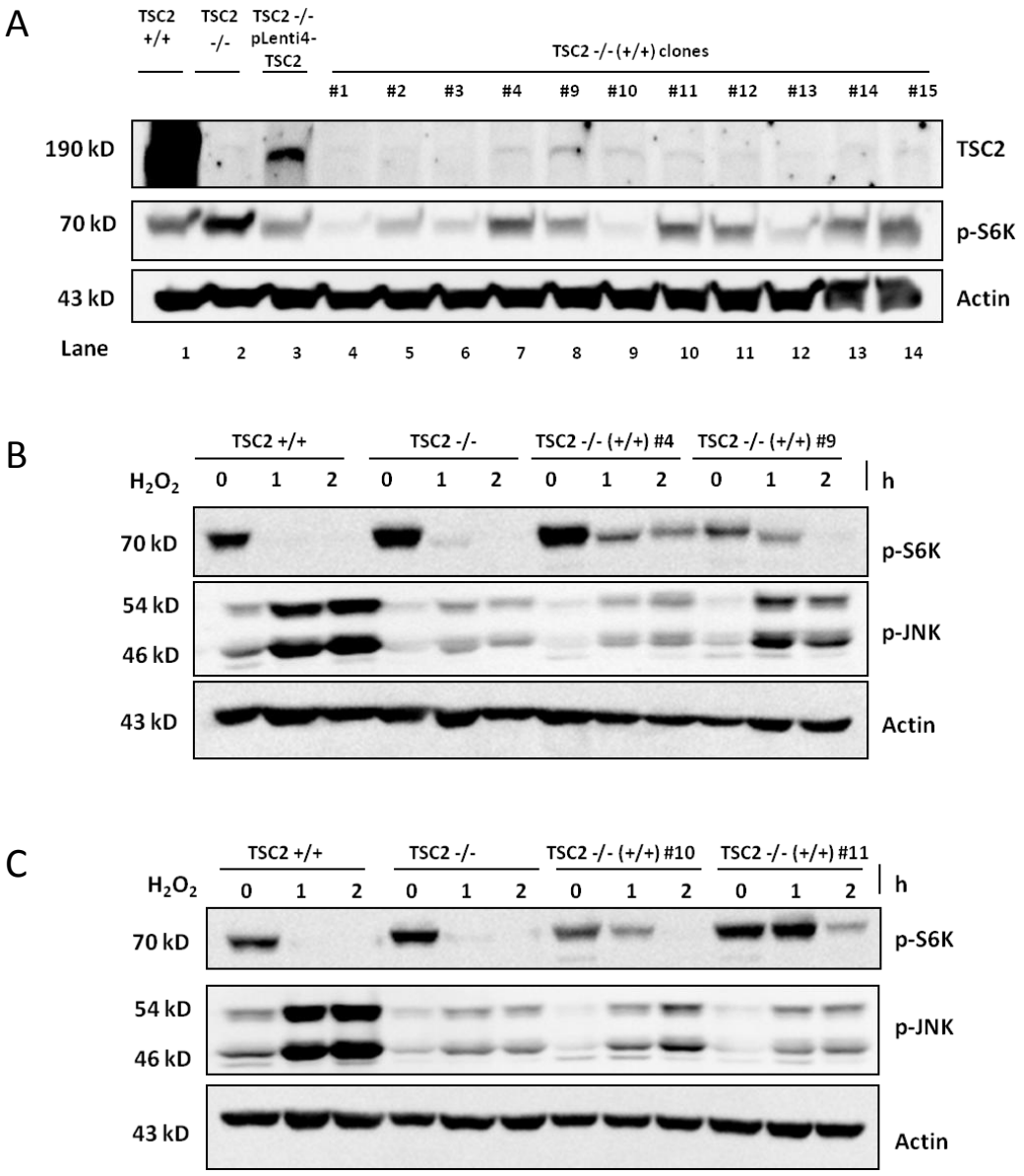


Figure 4.7 Reconstitution of *TSC2* increases activation of oxidative stress-induced JNK activation. (A) Generation of stable reconstituted *TSC2* in $TSC2^{-/-}$ MEFs. $TSC2^{+/+}$ (lane 1) and $TSC2^{-/-}$ (lane 2) cells served as controls respectively, while lane 3 was a transient expression of pLenti4-TSC2 plasmid in $TSC2^{-/-}$ cells for 48 hours. The clones labeled #1 to #4, and #9 to #15 in the subsequent lanes (lane 4 to 14) were stable cell lines of $TSC2^{-/-}$ MEFs reconstituted with *TSC2* (labeled as $TSC2^{-/-(+/+)}$. (B) and (C) Selection of positive clones for H_2O_2 treatment. The clones (labeled as #4, #9, #10, and #11) were further treated with H_2O_2 (0.5 mM) for the indicated time to examine the *TSC2* reconstitution effect on JNK activation. Cell lysates were collected and subsequently used for western blotting. Actin served as the loading control.

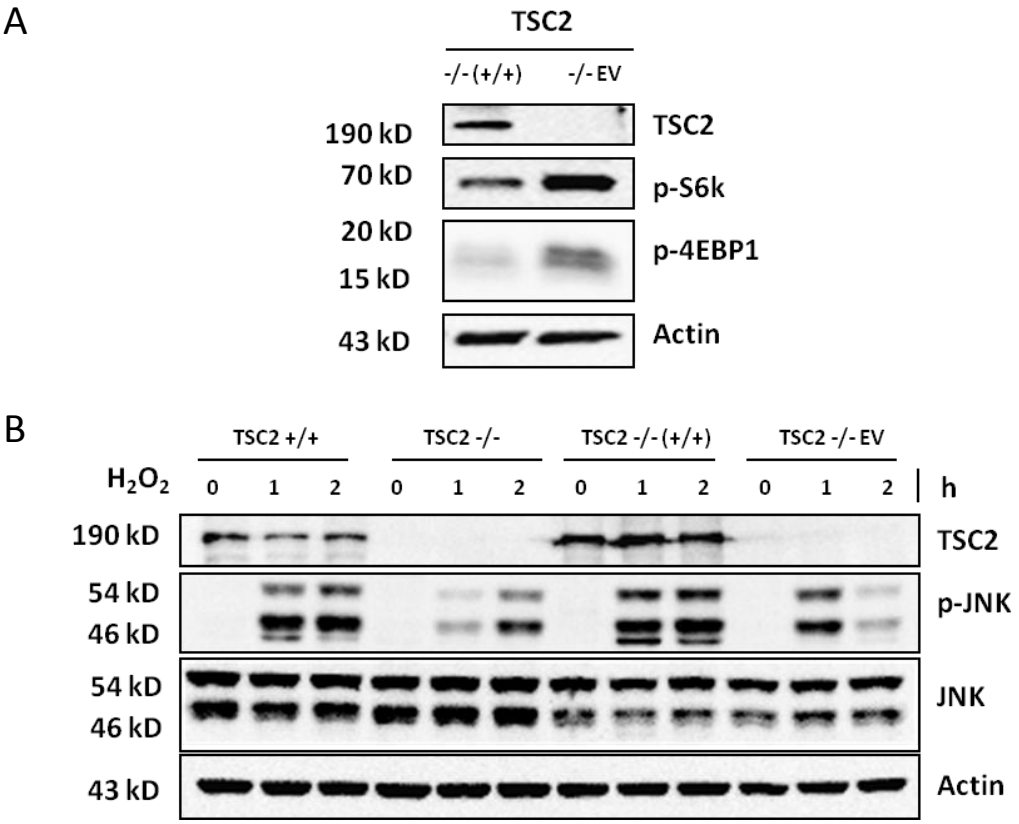


Figure 4.8 JNK activation is restored in *TSC2*-reconstituted stable cell line. (A) The pair of *TSC2* reconstituted stable cell line; $TSC2^{-/-(+/+)}$ and $TSC2^{-/-}$ empty vector, $TSC2^{-/-(EV)}$ (Huang et al., 2009a). The lysates of the *TSC2* reconstituted cells were collected after overnight incubation in full medium. (B) *TSC2* reconstitution restores JNK activation in response to H_2O_2. The $TSC2^{+/+}$, $TSC2^{-/-}$ as well as cells in panel A were treated for the indicated time with H_2O_2 (0.5 mM). Cell lysates were then analysed with western blot for the indicated markers. Actin was used as a loading control.

reconstituted cells was confirmed by the presence of TSC2 protein and the reduced p-S6K and p-4EBP1 level (Figure 4.8A). Consistently, H_2O_2-induced JNK activation was significantly higher in the reconstituted $TSC2^{-/-(+/+)}$ as compared to the $TSC2^{-/-(EV)}$ cells (Figure 4.8B). These results were comparable to the $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs used in this study, showed in the same western blot (Figure 4.8B). Taken together, these data clearly demonstrate that TSC2 is an important positive regulator for oxidative stress-mediated JNK signaling.

4.2.3 Autophagy pathway is independent of JNK-impairment signaling of $TSC2^{-/-}$ cells

As the earlier part of this thesis showed that $TSC2^{-/-}$ cells have a lower basal and inducible autophagy level (Figure 3.4 and 3.5), we sought to determine if lower autophagy plays a role in the impaired JNK activation in $TSC2^{-/-}$ cells. We first utilised choloroquine (CQ) to block lysosomal function and autophagy. As shown in Figure 4.9A and 4.9B, respectively, CQ had no effect on JNK activation in response to H_2O_2 and SNP in both $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs. Increased levels of LC3-II and p62 served as autophagic flux markers, as described earlier. Next, we used another autophagy inhibitor- wortmannin, which is known to block autophagy via suppression of PI3K Class III (Arcaro and Wymann, 1993; Wu et al., 2010). As shown in Figure 4.10, wortmannin markedly inhibited H_2O_2-induced JNK activation in both $TSC2^{+/+}$ and $TSC2^{-/-}$ cells, suggesting that suppression of autophagy fails to have any impact on JNK activation. To further rule out the involvement of autophagy in JNK activation, we treated WT MEFs and $Atg7^{-/-}$ MEFs with H_2O_2 and SNP. Atg7 acts as E1-like enzyme that is required for the conjugation phosphatidylethanolamine (PE) to LC3-I to form LC3-II protein (Mizushima et al., 2011). As expected, $Atg7^{-/-}$ MEFs were marked with absence of LC3-II and elevated level of p62 protein (Figure 4.11A). Interestingly, we observed a slightly increased level of JNK activation in $Atg7^{-/-}$ MEFs treated with H_2O_2 (Figure 4.11B) and SNP (Figure 4.11C). Therefore, it is believed that the impaired JNK activation observed in $TSC2^{-/-}$ cells is unlikely due to the lower autophagy level.

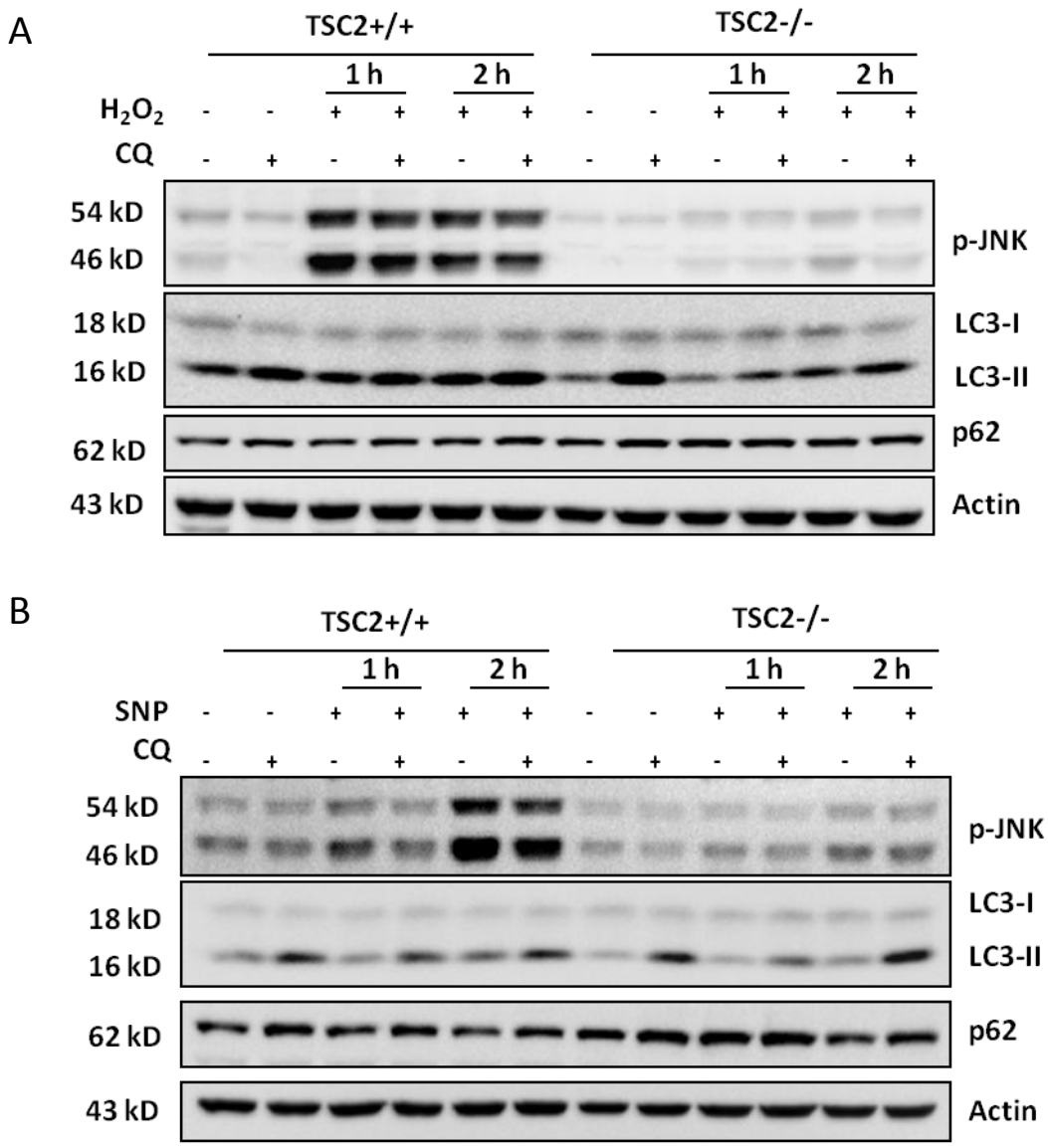


Figure 4.9 Inhibition of autophagy with chloroquine does not inhibit JNK signaling. (A) and (B) Effect of chloroquine (CQ) in JNK signaling. $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were treated with H_2O_2 (0.5 mM) or SNP (0.5 mM) with or without CQ (10 μM) for the indicated period of time. CQ was pretreated 1 hour prior to treatment. The cell lysates were subsequently collected for analysis by western blotting.

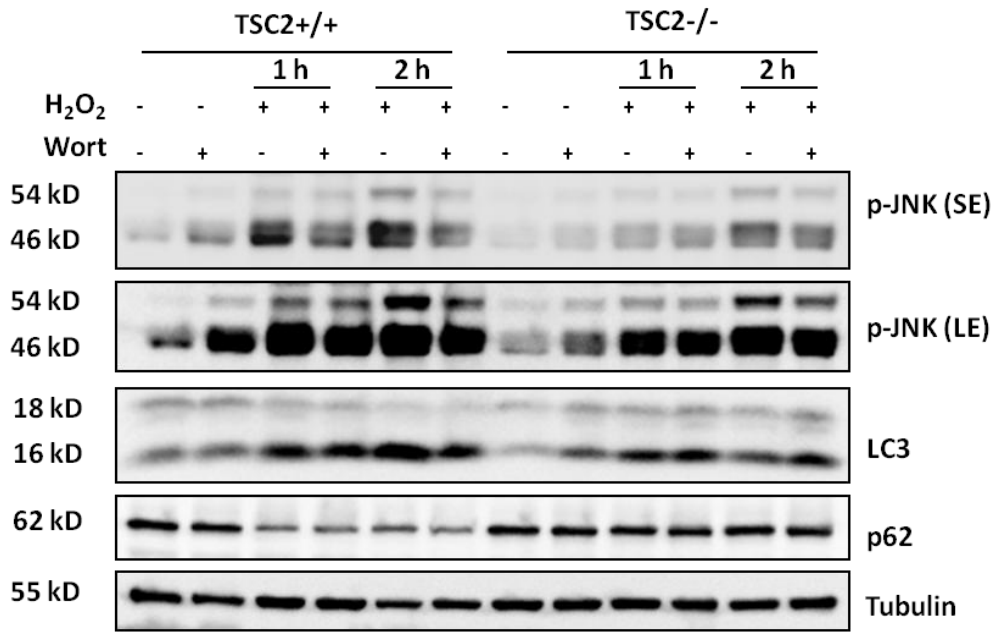


Figure 4.10 Inhibition of autophagy with wortmannin alters JNK signaling. H_2O_2 (0.5 mM) was added with or without wortmannin (Wort, 100 nM) for the indicated time. Wortmannin was pretreated for 1 hour prior to the addition of H_2O_2. Cell lysates were collected and western blotting was performed using the indicated antibodies. The reduction of JNK signaling was observed using shorter exposure (SE) or longer exposure (LE) of imaging system to compare the effect between both types of cells. Tubulin served as loading control.

A

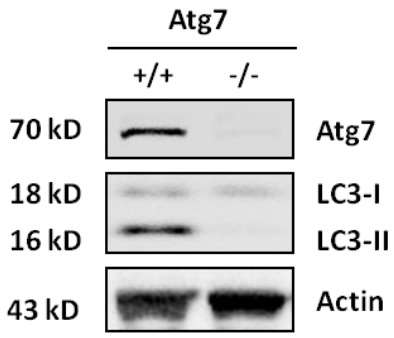
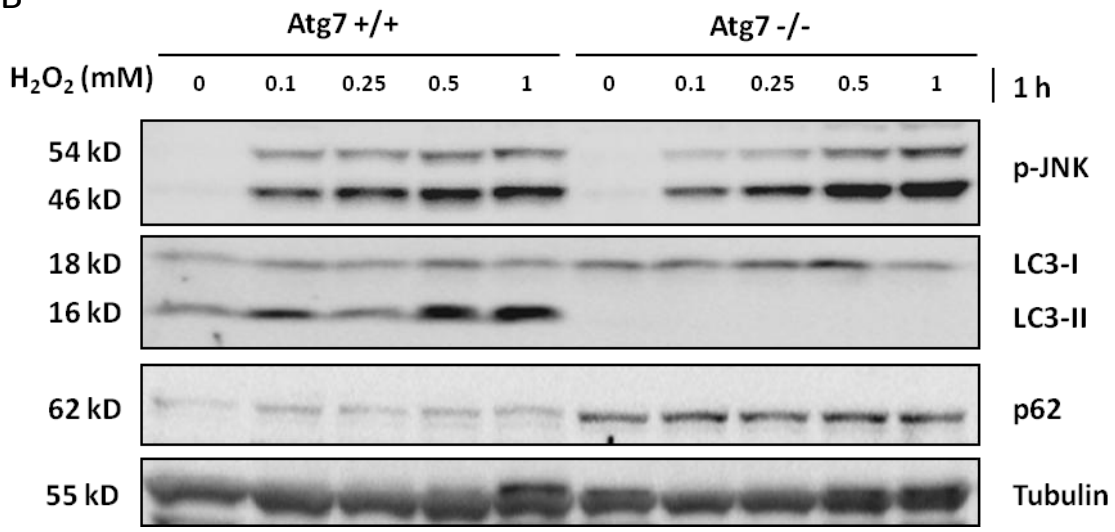
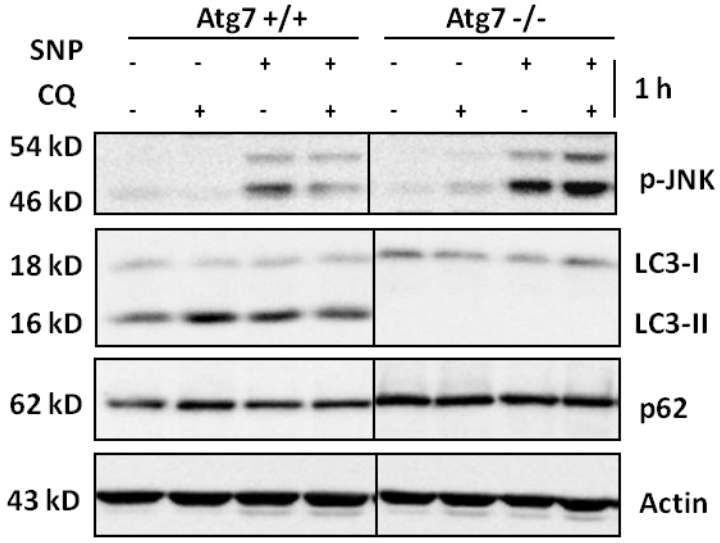


Figure 4.11 Autophagy-deficient Atg7-null cells do not show JNK impairment. (A) $Atg7^{+/+}$ and $Atg7^{-/-}$ MEFS. The cell lysates were collected after overnight incubation in full medium. (B) (next page) Dose-dependent effect of H_2O_2 in Atg7 cells. The Atg7 cells were treated with different doses of H_2O_2 as described earlier for 1 hour. (C) (next page) Effect of nitric oxide in Atg7 cells. Atg7 cells were treated with SNP (0.5 mM) with or without CQ, as described earlier in Figure 4.9B. Cell lysates were collected and subsequently analysed with western blotting for the indicated markers. Actin and tubulin served as loading controls for respective panels.

B



C



4.2.4 Impaired JNK activation in TSC2$^{-/-}$ cells is not associated with constitutively active mTORC1

As TSC2$^{-/-}$ cells have higher basal mTORC1 activity (Jaeschke et al., 2002), we next investigated the role of mTORC1 signaling in H_2O_2-induced JNK activation using various inhibitors of the PI3K-Akt-mTORC1 pathway, including LY294002, rapamycin and PP242. All these three inhibitors (LY294002,

rapamycin, and PP242) were able to effectively abolish the aberrant mTORC1 activity in $TSC2^{-/-}$ cells, evidenced by the absence of p-S6K in both $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs (Figure 4.12A-4.12C). Interestingly, all the three inhibitors failed to affect H_2O_2-mediated JNK activation in either the $TSC2^{+/+}$ or $TSC2^{-/-}$ MEFs. Notably, H_2O_2 treatment alone also effectively suppressed mTORC1 in both $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs, being consistent with the earlier reports (Li et al., 2010; Smith et al., 2005). Such data thus indicate that hypersensitivity of mTORC1 is not directly involved in H_2O_2-mediated impaired JNK activation seen in $TSC2^{-/-}$ MEFs.

As raptor is the regulatory subunit of mTORC1 (Chi, 2012), we next took a genetic approach by knocking down raptor in both $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. As shown in Figure 4.13A, the successful knockdown of raptor significantly reduced the mTORC1 activity in $TSC2^{+/+}$ cells, evidenced by the reduction of p-S6K and an increase of p-Akt. Similarly in $TSC2^{-/-}$ cells, raptor knockdown inhibited the constitutive mTORC1 activity (Figure 4.13B). However, raptor knockdown did not cast any effect on JNK activation in both type of cells. Taken together, it is believed that the impaired JNK activation in $TSC2^{-/-}$ cells is not associated with the constitutively activated mTORC1.

4.2.5 Upstream MAPK kinases are independent of JNK-impairment signaling in $TSC2^{-/-}$ cells

In this part of our study, we attempted to examine and compare the changes of upstream MAPK kinases between $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. H_2O_2 is known to activate JNK through three phosphorylation cascades comprising of

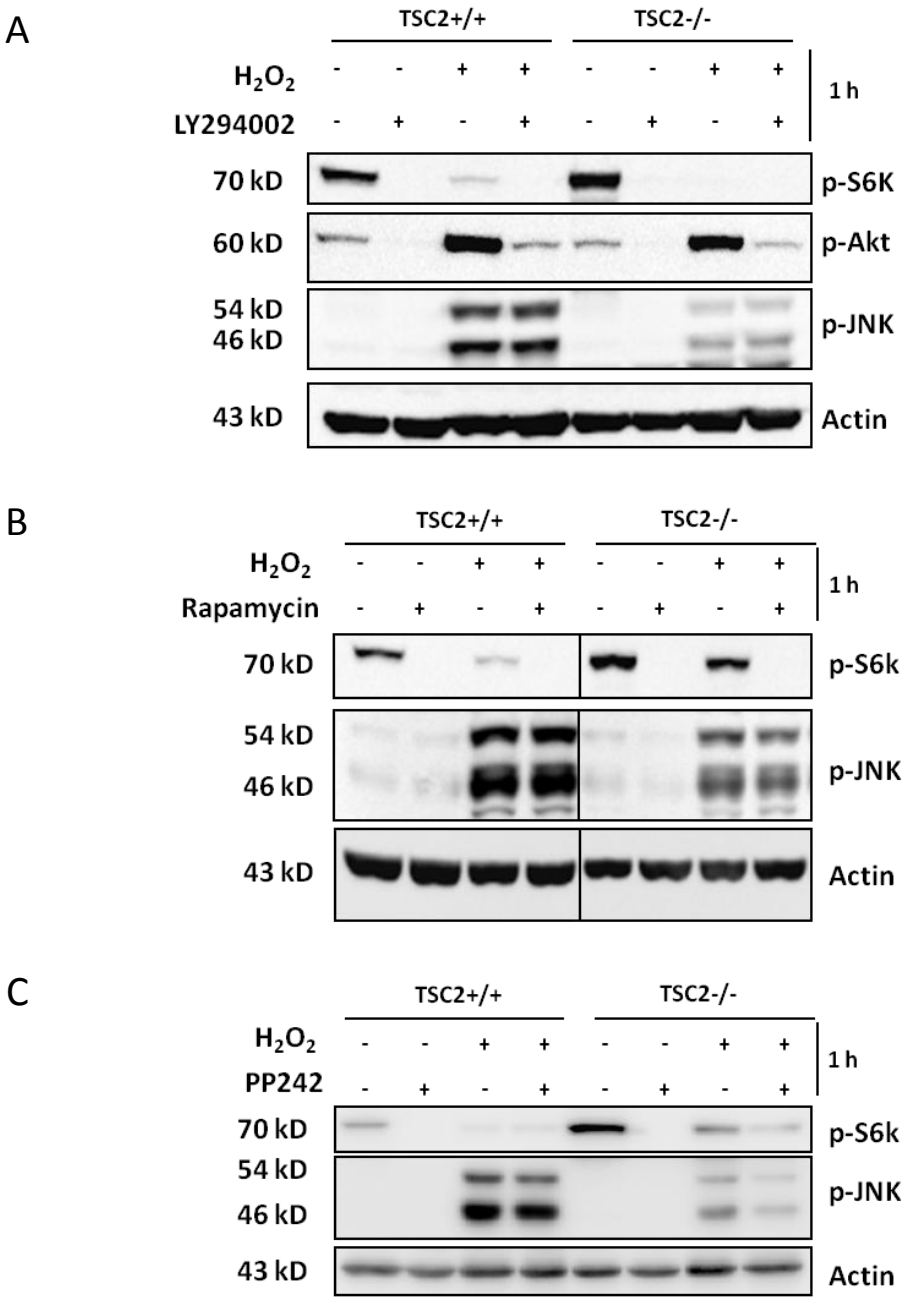


Figure 4.12 JNK impairment in TSC2$^{-/-}$ cells is not due to PI3K-Akt-mTORC1 pathway. (A) PI3K inhibitor does not affect JNK signaling. TSC2 MEFs were treated with H_2O_2 (0.5 mM) with or without PI3K-Akt inhibitor, LY294002 (10 μM) for the indicated time. The mTORC1 inhibitors, (B) Rapamycin or (C) PP242 does not further upregulate JNK signaling in TSC2$^{-/-}$ cells. Rapamycin (10 nM) or PP242 (1 μM) were added respectively into the cells, with or without H_2O_2 (0.5 mM) for 1 hour. Pretreatment with respective inhibitors for 1 hour was performed prior to the addition of H_2O_2. Cells were lysed and cell lysates were analysed with western blotting using the indicated antibodies. Actin served as a loading control.

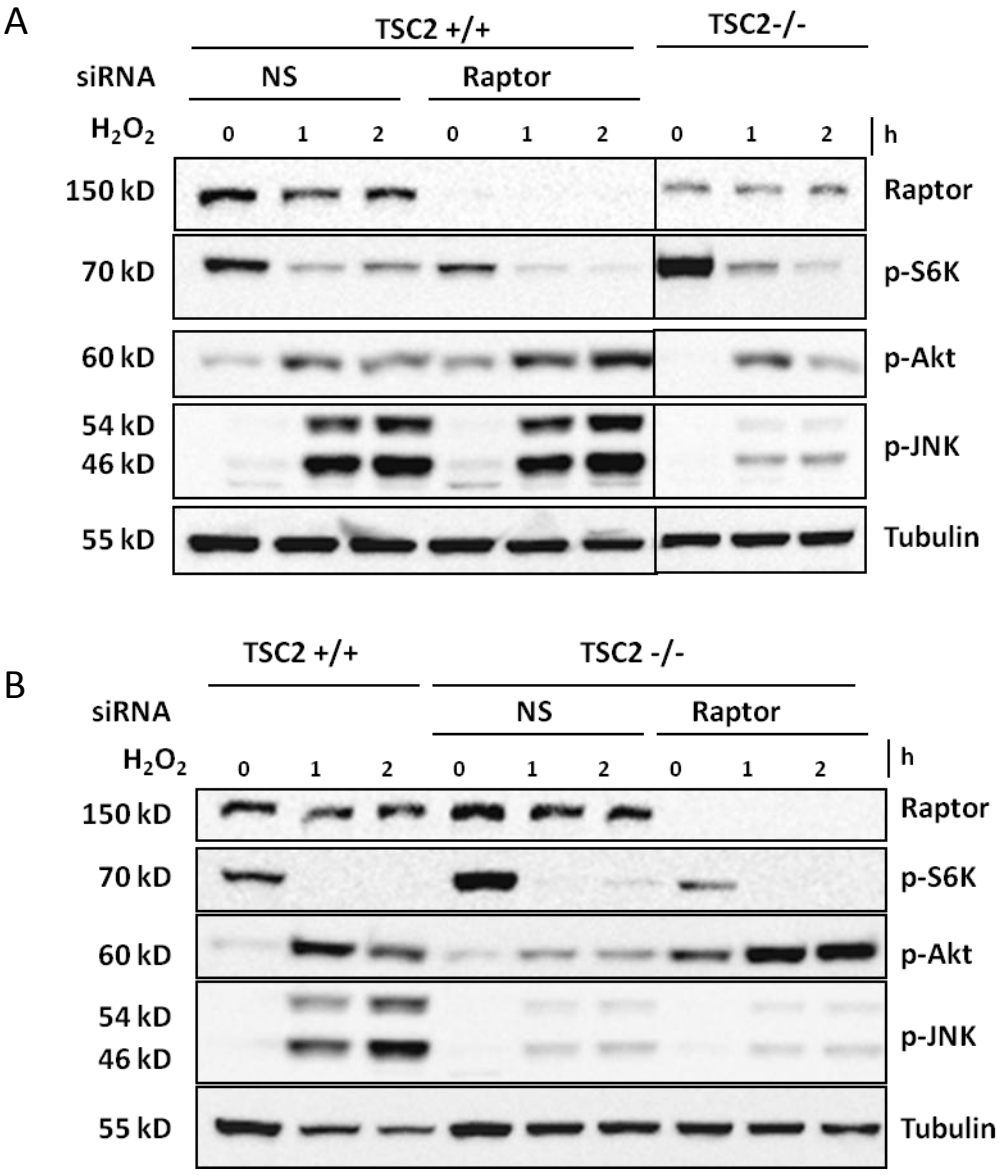


Figure 4.13 Raptor knockdown does not affect JNK signaling. (A) Effect of raptor knockdown in $TSC2^{+/+}$ cells. Non-specific (NS) siRNA that served as control or raptor siRNA knockdown in $TSC2^{+/+}$ cells was performed. After 48 hours, cells were treated with H_2O_2 (0.5 mM) for the indicated time. (B) Effect of raptor knockdown in $TSC2^{-/-}$ cells. Similarly as described in panel A, knockdown of NS or raptor was performed in $TSC2^{-/-}$ cells. The pair of TSC2 cells were treated simultaneously with respective cells of siRNA-knockdown in both panels. Cell lysates were subsequently analysed with western blotting for the indicated markers. Tubulin served as loading control.

MAPKKK (MAP3K), MAPKK (MAP2K) and MAPK (Chang and Karin, 2001; Pearson et al., 2001). The activation of apoptosis signal-regulating kinase 1

(ASK1), a MAP3K, which is a key upstream kinase in response to H_2O_2, while the activated ASK1 will then phosphorylate and activate MKK4 and MKK7, the two key MAP2K for JNK activation (Liu et al., 2007). As shown in Figure 4.14, H_2O_2 exerted similar effects on ASK1 (Thr845) and MKK4 (Ser257) between TSC2 $^{+/+}$ and TSC2$^{-/-}$ cells, suggesting that the impaired JNK activation in TSC2$^{-/-}$ cells is unlikely caused by defective upstream kinases. In addition, we also examined the changes of the other two MAPK pathways: p38 and ERK. Interestingly, there were no evident differences in the phosphorylation level of MKK3/MKK6 and p38 between these two types of cells when treated with H_2O_2 (Figure 4.14).

However, there was a reduction in both ERK (p-ERK Thr202/Tyr204) and a slight downregulation of MKK4 at T261 (p-MKK4 Thr261) phosphorylation level in TSC2$^{-/-}$ cells when compared to TSC2$^{+/+}$ cells (Figure 4.14). These observations suggested that the upstream regulation of MAPK is unlikely to be the main mechanism responsible for the impaired JNK activation in TSC2$^{-/-}$ cells. Due to the unavailability of a good antibody, we were thus limited in obtaining a functional immunoblotting for MKK7 phosphorylation. However, we have performed an overexpression study using JNKK2 (also known as MKK7)-JNK1 plasmid that displays constitutive JNK activation (Zheng et al., 1999), which will be discussed in the next section (Figure 4.18).

4.2.6 TSC2$^{-/-}$ cells have a significantly lower level of tyrosine phosphorylation

Based on the well-known fact that JNK is phosphorylated at both tyrosine

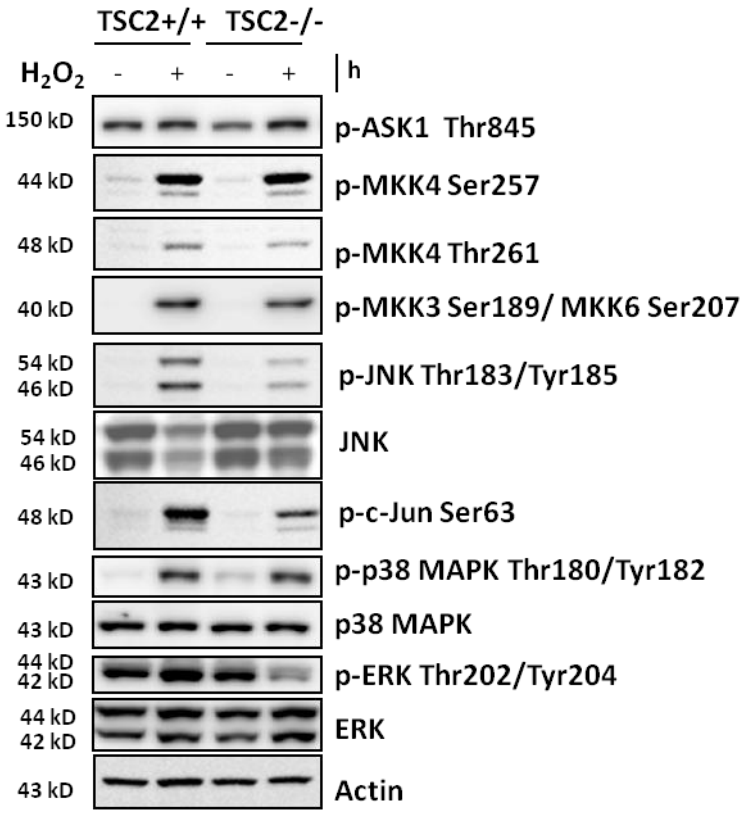


Figure 4.14 Upstream of MAPK signaling (MAP3K-MAP2K) is not downregulated in $TSC2^{-/-}$ cells. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were treated with H_2O_2 (0.5 mM) for 1 hour. Cell lysates were collected and western blotting was performed for the indicated antibodies. Actin was the loading control.

and threonine residues, we asked if the either of these two signaling is affected in $TSC2^{-/-}$ cells. We first examined the tyrosine and threonine phosphorylation states in TSC2 cells treated with H_2O_2. We observed a significant decrease of tyrosine phosphorylation in $TSC2^{-/-}$ cells in response to H_2O_2, while the phosphorylated threonine level was also slightly reduced, but to a much lesser extent (Figure 4.15A). Next, we used okadaic acid (OA), a general serine/threonine phosphatase inhibitor (Garcia et al., 2002) and sodium orthovanadate (Na_3VO_4), which is a general tyrosine phosphatase inhibitor (Kim et al., 1999) to inhibit phosphatases under basal conditions. As expected, there were higher phosphorylated levels of

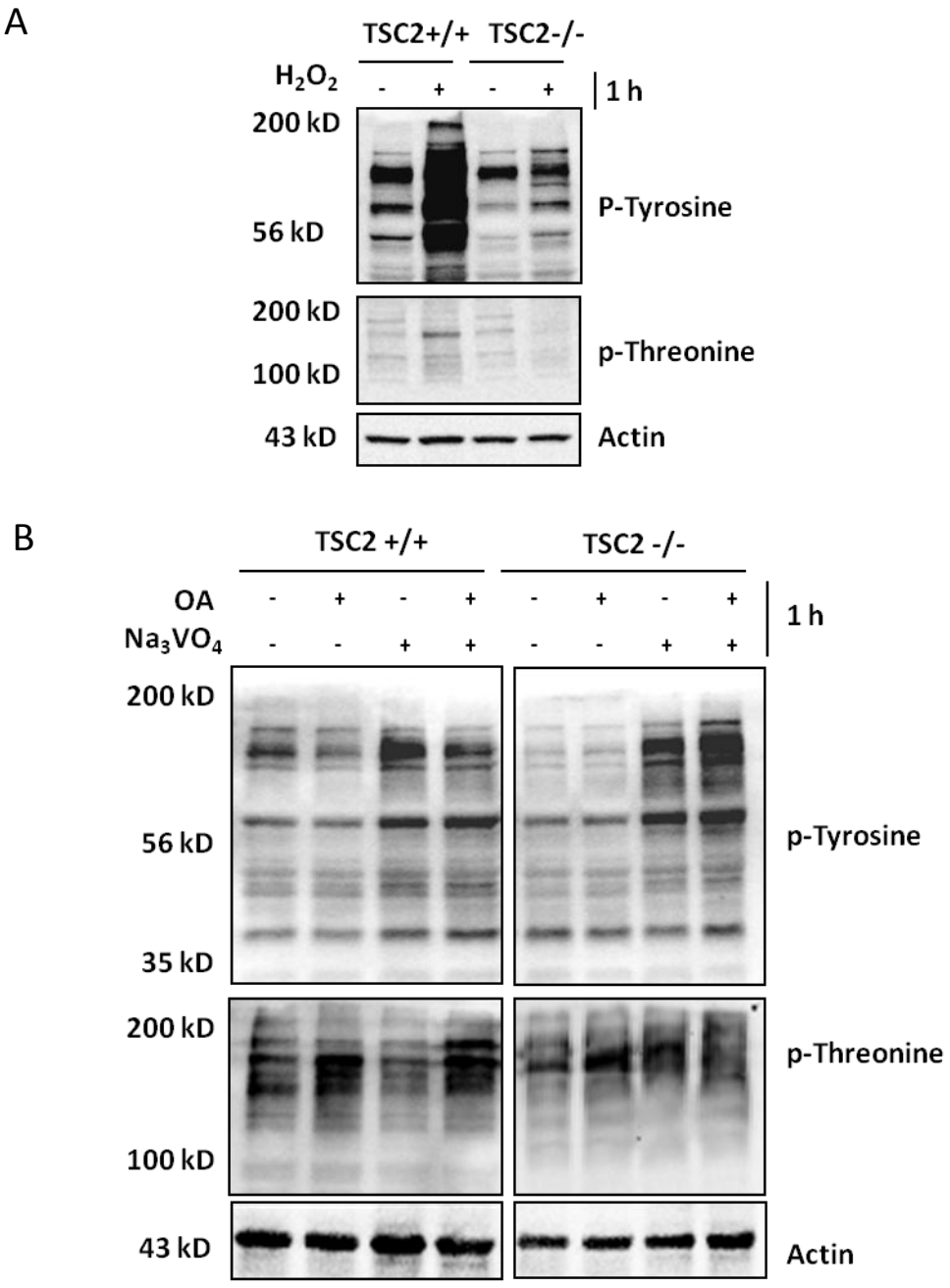


Figure 4.15 Tyrosine phosphorylation activity is significantly lower in $TSC2^{-/-}$ MEFs. (A) Tyrosine phosphorylation is impaired in $TSC2^{-/-}$ cells. The pair of TSC2 MEFs were treated with H_2O_2 (0.5 mM) for 1 hour. (B) Effect of tyrosine and serine/threonine phosphatase inhibitors. TSC2 MEFs were treated with phosphatase inhibitors okadaic acid (OA, 100 nM) or sodium orthovanadate (Na_3VO_4, 100 µM), or both for 1 hour. Western blotting was subsequently performed with the collected cell lysates to blot for the indicated markers. Actin served as the loading control.

tyrosine and threonine, respectively, in $TSC2^{-/-}$ cell with the addition of these two inhibitors (Figure 4.15B).

We next tested if protein phosphatases are involved in the impaired JNK activation in $TSC2^{-/-}$ cells. Results showed that while OA had not much effect on p-JNK level in the $TSC2^{-/-}$ cells, the addition of orthovanadate significantly restored the JNK signaling in $TSC2^{-/-}$ cells in response to H_2O_2 (Figure 4.16A). Such observations thus suggest that the impaired JNK activation in TSC2-null cells is likely to be caused by a higher activity of tyrosine phosphatases. Similar trend was observed when cells were treated with both TNFα and UV (Figure 4.16B and 4.16C, respectively).

Notably, while comparing between the $TSC2^{+/+}$ and $TSC2^{-/-}$, different restoration activity of JNK signaling seemed to be observed in the three panels of Figure 4.16. In panel A, the JNK restoration of H_2O_2+Na_3VO_4 treatment was significantly higher in $TSC2^{-/-}$ MEFs while less effect was observed in $TSC2^{+/+}$ cells whereas in both panel B and C (Figure 4.16), the effect of orthovanadate phosphatase inhibitor was significantly higher in the $TSC2^{+/+}$ MEFs. One possible reason for this observation is that the MKPs that were involved could possibly be highly expressed and activated in response to H_2O_2 in the TSC2-deficient cells. Moreover, it is also noteworthy to note that the treatment of H_2O_2 was longer (1 hour) while the TNFα and UV treatments were shorter, at a 20 minutes time point. It is thus possible that in cells with functional TSC, the deactivation of MKPs involved may have already taken place at the longer time point.

To further confirm the possible involvement of tyrosine phosphatases in

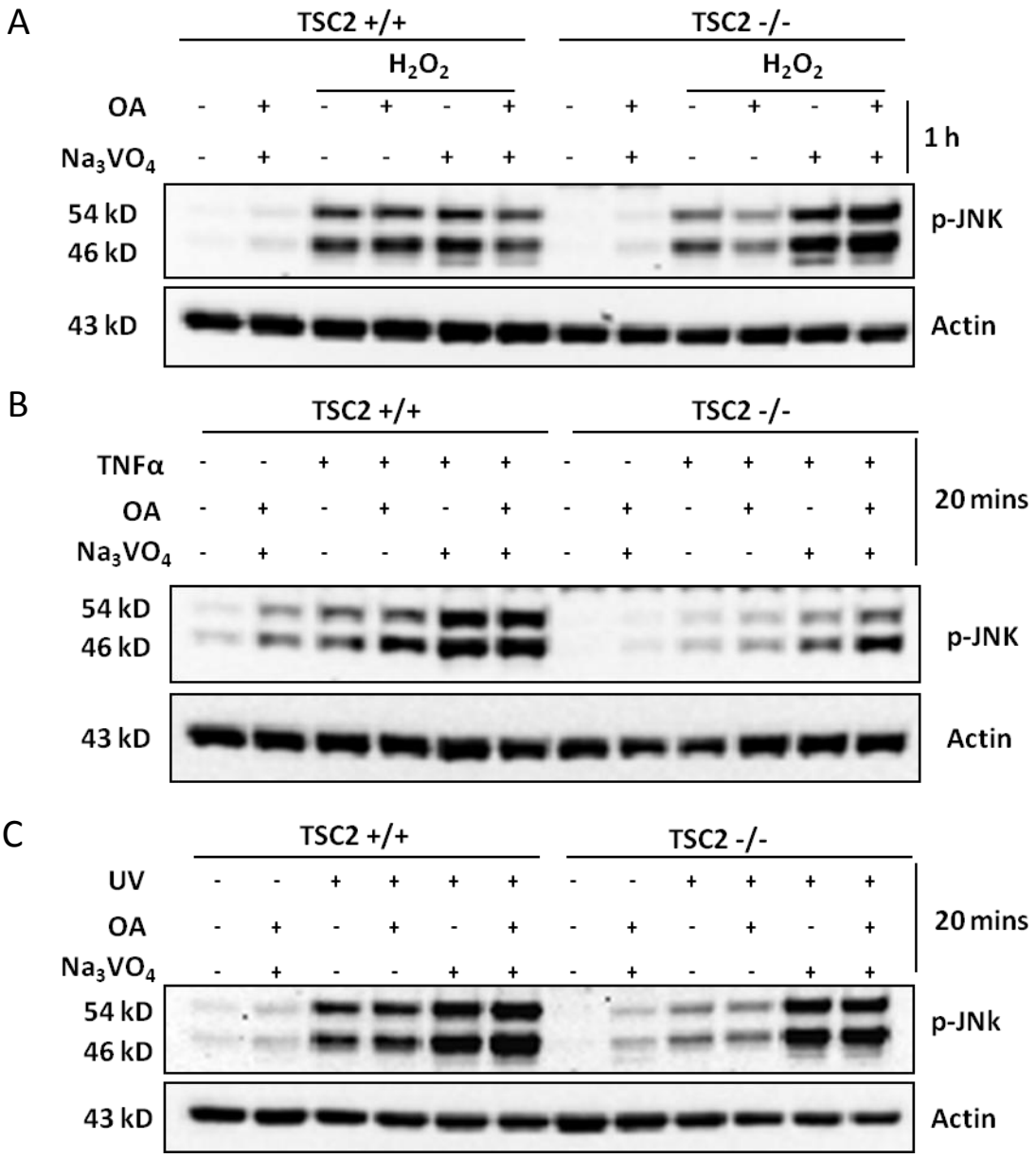


Figure 4.16 Tyrosine phosphatase inhibitors restored JNK activation in the $TSC2^{-/-}$ cells. (A) Effect of phosphatase inhibitors on H_2O_2. $TSC2^{+/+}$ and $TSC2^{-/-}$ were pre-treated with the phosphatase inhibitors, okadaic acid (OA, 100 nM) and sodium orthovanadate (Na_3VO_4, 100 µM), or both for 1 hour prior to the addition of H_2O_2 (0.5 mM) as indicated. (B) Effect of phosphatase inhibitors on TNFα. The MEF cells were treated with TNFα (10 ng/ml) as described in panel A for 20 minutes. (C) Effect of phosphatase inhibitors on UV radiation. Cells were radiated with UV as described in panel A for 20 minutes. Subsequently, western blot was performed using cell lysates and immunoblotted for the indicated markers. Actin was the loading controls.

H_2O_2-mediated JNK activation, we utilised the stable *TSC2*-reconstituted cells. As expected, reconstitution of *TSC2* markedly reduced the tyrosine phosphorylation level in response to H_2O_2 when compared to their corresponding counterparts. Meanwhile, no evidence difference was found for threonine phosphorylation (Figure 4.17), suggesting the possibility that TSC2 protein specifically affects tyrosine phosphorylation. To further strengthen our hypothesis, we utilised the plasmid expressing a constitutive active level of JNK, 3HA-JNKK2-JNK1 that contains the JNKK2-JNK1 fusion protein (Zheng et al., 1999) and examined the tyrosine phosphorylation states in response to H_2O_2. Consistently, we found that the immunoprecipitated JNKK2-JNK1 in $TSC2^{-/-}$ cells had a much lower tyrosine phosphorylation level as compared to the counterpart in the wild type cells (Figure 4.18), further implying a TSC-mediated role in tyrosine phosphatase activity.

4.2.7 TSC2 regulates MKP-1 expression

Having the earlier observations that the H_2O_2-induced tyrosine phosphorylation was poorly activated in $TSC2^{-/-}$ cells, we next sought to determine if the MAPK phosphatases (MKPs) are involved in the impairment of the JNK signaling in $TSC2^{-/-}$ cells under oxidative stress. JNK is dephosphorylated by MKPs, a group of specialised phosphatases acting specifically on tyrosine and threonine residues for MAPK (Karin and Gallagher, 2005). The important MKPs that regulate JNK activity include MKPs-1, -3, -5 and -7 (Hirsch and Stork, 1997; Kamata et al., 2005; Masuda et al., 2001; Sanchez-Perez et al., 2000; Tanoue et al., 2001; Theodosiou et al., 1999),

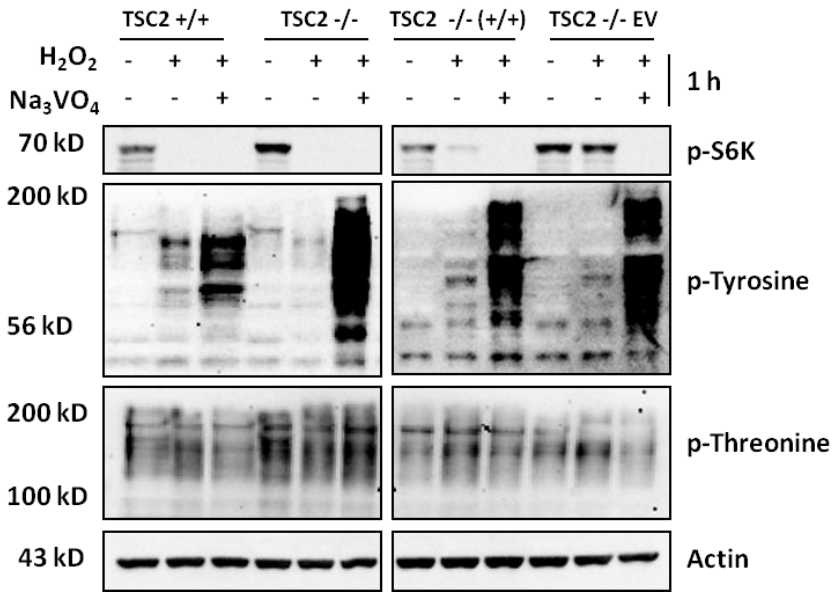


Figure 4.17 Tyrosine phosphatase inhibitor significantly upregulates tyrosine phosphorylation in TSC2-null (EV) cells. $TSC2^{+/+}$, $TSC2^{-/-}$, as well as reconstituted $TSC2^{-/-(+/+)}$ and $TSC2^{-/-(EV)}$ were treated with sodium orthovanadate (Na_3VO_4, 100 μM), or both for 1 hour prior to the addition of H_2O_2 (0.5 mM). Subsequently, collected cell lysates were analysed with western blot for the indicated markers.

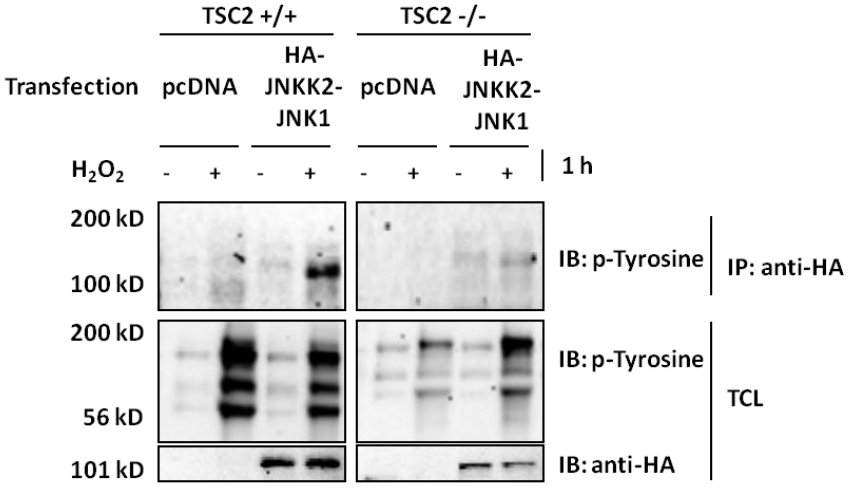


Figure 4.18 Immunoprecipitated phospho-tyrosine is lower in $TSC2^{-/-}$ cells transfected with constitutive active JNKK2-JNK1 fusion protein. $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs, respectively were transfected with empty vector (pcDNA) or HA-JNKK2-JNK1 plasmid. After transfected for 48 hours, the cells were treated with H_2O_2 (0.5 mM) for 1 hour. The total phosphorylated-tyrosine immunoprecipitated (IP) with anti-HA antibody was immunoblotted (IB) by western blot. Other markers in the total cell lysates (TCL) was similarly obtained from western blotting.

although it was reported that MKP-3 is more specific for ERK deactivation (Bermudez et al., 2011; Keyse, 2008). Thus, we examined the changes of MKP-1,-3, -5 and -7 mRNA levels in H_2O_2-treated cells. We noticed that MKP-1 mRNA level was upregulated significantly in $TSC2^{-/-}$ cells when compared to the $TSC2^{+/+}$ cells (Figure 4.19).

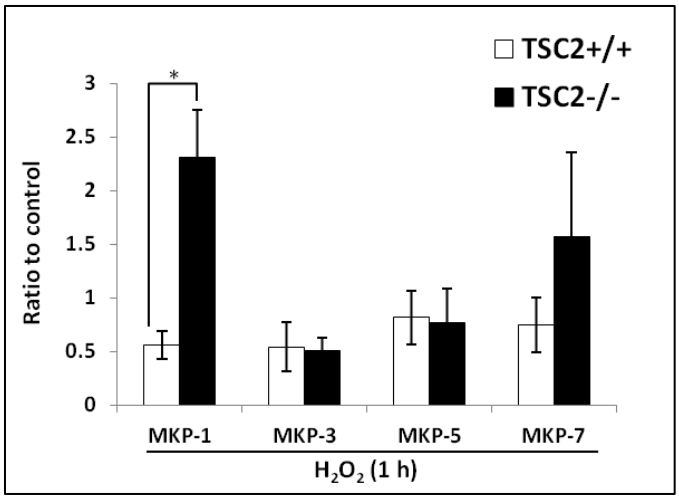


Figure 4.19 MKP-1 mRNA level is higher in $TSC2^{-/-}$ cells. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were treated with H_2O_2 (0.5 mM, 1 hour) and then the total RNA of all indicated samples were isolated. RNA was reverse-transcribed to cDNA using PCR, followed by real-time PCR (RT-PCR) amplification. The MKPs data obtained from RT-PCR were expressed as folds relative to respective control. Respective *GAPDH* in each group was used as the internal control for $TSC2^{+/+}$ and $TSC2^{-/-}$. Statistical significance (*p<0.05, *t*-test) of MKP-1 level in respective treatments was indicated in the bar chart.

We further studied the interaction of TSC2 to MKP-1 by performing co-immunoprecipitation assay. In $TSC2^{+/+}$ MEFs that were transfected with Flag-MKP-1, there was an increased binding of TSC2 to MKP-1 in response to H_2O_2 treatment (Figure 4.20). Interestingly, while there was an increase of TSC2 protein, a substantial amount of MKP-1 protein level was reduced in the treated cells. This indicated that TSC2 is likely to suppress the function of MKP-1. Taken

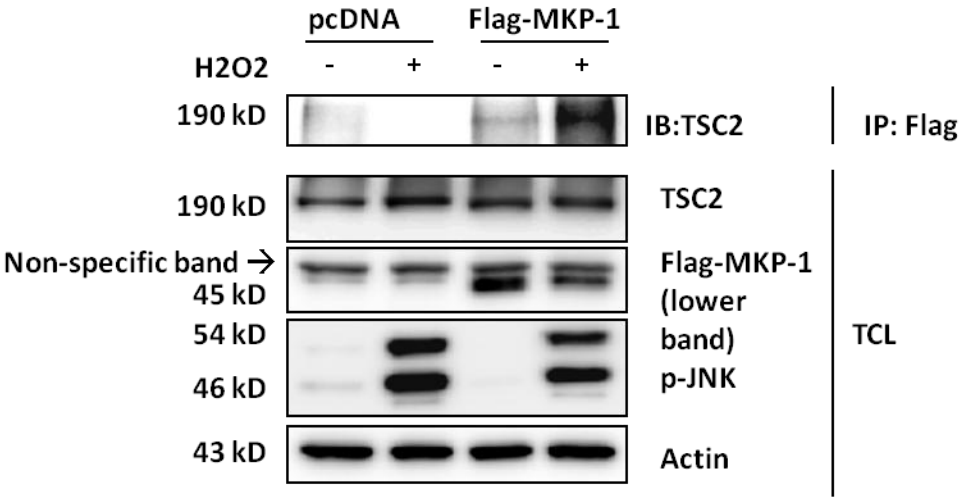


Figure 4.20 TSC2 binds to MKP-1. $TSC2^{+/+}$ MEFs was transfected with Flag-MKP-1 for 48 hours, followed by treatment of H_2O_2 (0.5 mM) for 1 hour. The total TSC2 immunoprecipitated (IP) with anti-Flag antibody was immunoblotted (IB) by western blot. Other markers in the total cell lysates (TCL) were similarly obtained from western blotting. Actin was the loading control.

together, data from this part of our study suggest that TSC2 protein regulates at least one of the MKPs (MKP-1) via protein-protein interaction. Absence of TSC2 may lead to higher expression of MKP-1 which then impairs JNK activation in the $TSC2^{-/-}$ cells.

4.2.8 JNK impairment sensitizes $TSC2^{-/-}$ cells to necrosis

To investigate the physiological and pathological relevance of the TSC2-MKP1-JNK signaling pathway identified above, we further tested whether JNK impairment in $TSC2^{-/-}$ cells contributes to the susceptibility of $TSC2^{-/-}$ cells to stress-mediated cell death. We found that the specific JNK inhibitor, SP600125, failed to protect $TSC2^{-/-}$ cells against H_2O_2-induced cell death (Figure 4.21A). This observation can be understood as the JNK activation is in fact, already low in the $TSC2^{-/-}$ MEFs.

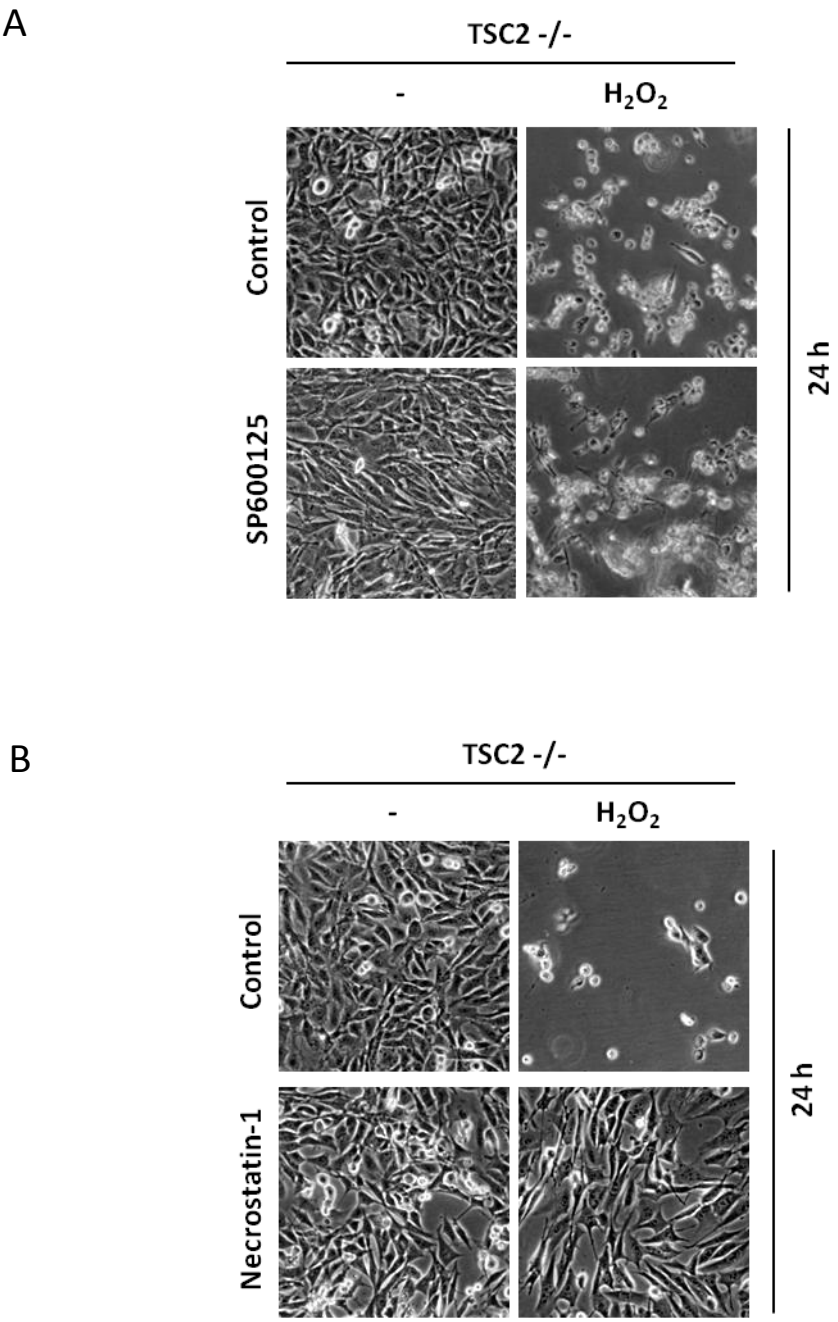
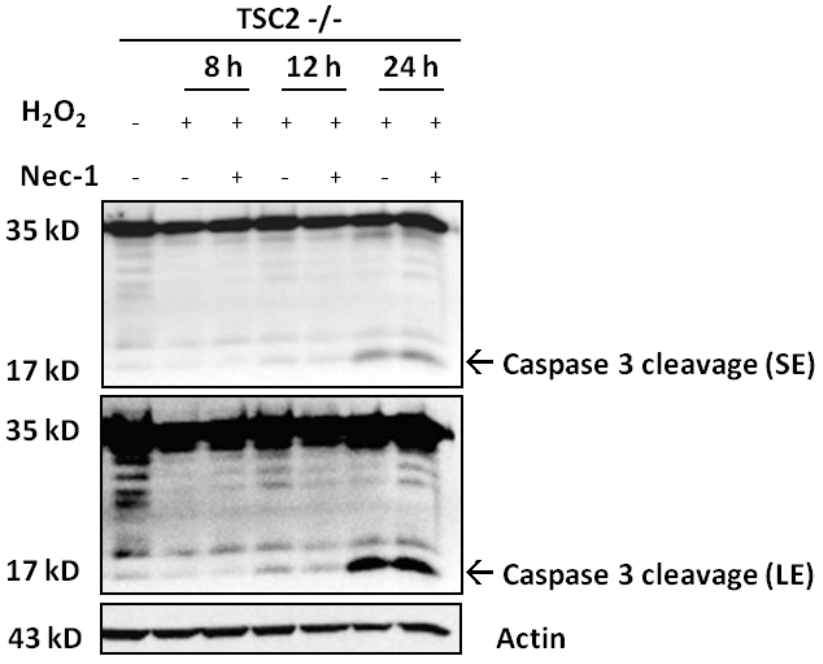


Figure 4.21 H_2O_2-induced cell death in $TSC2^{-/-}$ MEFs. (A) JNK inhibitor, SP600125 does not protect $TSC2^{-/-}$ cells from H_2O_2-induced cell death. TSC2 cells were treated with H_2O_2 (0.5 mM) with or without JNK inhibitor with SP600125 (20 µM). (B) Necroptosis inhibitor, necrostatin-1 protected $TSC2^{-/-}$ from H_2O_2-induced cell death. Cells were treated as in panel A with or without necrostatin-1 (30 µM) for 24 hours. Pictures were examined and photographed at indicated time using an inverted microscope (X 100).

Meanwhile, it has been reported previously that JNK is able to promote necrotic cell death under oxidative stress (Zhang et al., 2007). Thus, we further investigated H_2O_2-induced cell death using necrostatin-1 (Nec-1), a specific necrosis inhibitor via suppression of RIP1 (Degterev et al., 2008). Surprisingly, treatment with Nec-1 effectively protected $TSC2^{-/-}$ against H_2O_2-induced cell death, evidenced by the morphological changes (Figure 4.21B) and cell viability determined by the PI-exclusion test (Figure 4.22B). Such observations thus indicate that H_2O_2 mainly induces necroptotic cell death in $TSC2^{-/-}$ cells. Intriguingly, we also observed caspase 3 cleavage in $TSC2^{-/-}$ cells treated with H_2O_2 which was not affected by the addition of Nec-1, suggesting the possibility that both apoptosis and necrosis co-exist in H_2O_2-treated $TSC2^{-/-}$ cells. Taken together, these data suggest that H_2O_2-induced necrosis in $TSC2^{-/-}$ cells are associated with the impaired JNK signaling.

A



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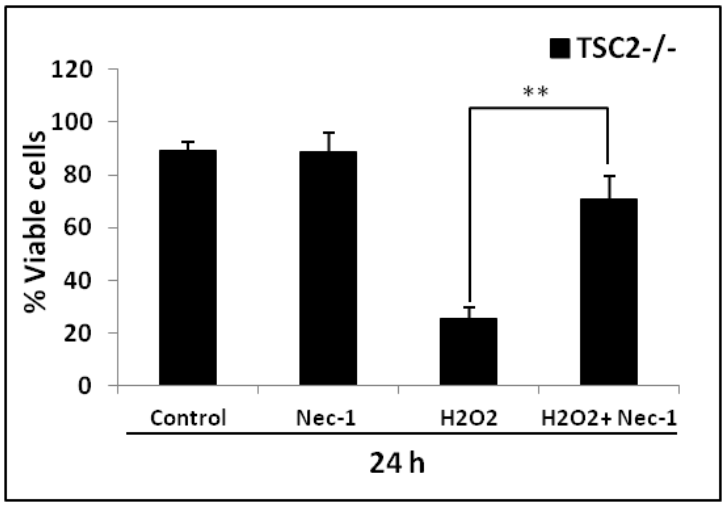

Figure 4.22 H_2O_2 induces apoptotic and necrotic cell deaths in $TSC2^{-/-}$ MEFs. (A) Apoptotic cells is present in $TSC2^{-/-}$ treated with H_2O_2 and necroptosis inhibitor. $TSC2^{-/-}$ MEFS were added with or without necrostatin-1 (Nec-1, 30 μM) in H_2O_2 (0.5 mM) -treated cells for the indicated period of time. Cell lysates were subsequently used for western blotting to blot for the indicated antibodies. Caspase 3 cleavage was still observed in necrostatin-1 treated cells, blotted for shorter exposure (SE) or longer exposure (LE) with imaging system. Actin was the loading control. (B) PI live exclusion staining for cell death quantification. The cells were treated as described in (A) for 24 hours. The percentage of viable cells was then measured with PI (5 μg/ml) using flow cytometry. All statistical data in all the figures are presented as means ± S.D of three independent experiments (**p<0.01, t-test).

4.3 Discussion

In this part of our study, we demonstrate that TSC protein possesses additional functions in mediating stress responses via regulation of JNK signaling and cell death. We provide evidence showing that TSC2 protein and the TSC1/TSC2 complex are able to promote oxidative stress-mediated JNK activation via engaging MKP-1. In cells deficient of TSC2 or TSC1, the JNK activation is impaired due to up-regulation of MKP-1, a process we found independent of mTORC1 activity. Our findings thus expand the functional scope of TSC and provide a novel link connecting TSC proteins to JNK signaling in

mediating oxidative stress and cell death.

TSC1 forms a heterodimer with TSC2 protein to negatively regulate downstream mTORC1 activity (van Slegtenhorst et al., 1998). Thus, in TSC1- or TSC2-deficient cells, the absence of TSC protein complex would lead to constitutive activation of mTORC1 (Jaeschke et al., 2002; Kwiatkowski et al., 2002), which is supported by our data as shown in Figure 3.1A. One key finding in our study was the impairment of JNK activation observed in both types of MEFs, which were either deficient of TSC1 or TSC2, despite of the various stimuli used (H_2O_2, TNFα, UV radiation and SNP) (Figure 4.1-4.4), thus strongly suggesting that the functional TSC1/TSC2 complex is involved in the activation of JNK signaling. Moreover, a recent study has shown that *p53* is involved in stimulating JNK activity under stress (Gowda et al., 2012). The TSC1 and TSC2 MEFs cells used in our study were known to be *p53* positive and negative, respectively (Kwiatkowski et al., 2002; Zhang et al., 2003a). As both TSC1- and TSC2-deficient cells showed similar trends with reduced JNK activation when stimulated (respective Figure 4.1, 4.2, 4.3, and 4.4), thus our study thus suggests that *p53* is unlikely to play an important role in JNK activation observed in these cells.

Another profound observation from our study was the independent roles of mTORC1 hyperactivation, autophagy and upstream MAP3K-MAP2K in the impaired JNK signaling of $TSC2^{-/-}$ cells. In Chapter 3, the hypersensitivity of $TSC2^{-/-}$ MEFs to various cell death stimuli was associated with a lower autophagy level, which was mainly due to the suppression of autophagy by aberrant

mTORC1 activity. However, in this study, we found that neither mTORC1 nor autophagy was involved in the impaired JNK activation in $TSC2^{-/-}$ MEFs (Figure 4.9-4.13). Such data could be understood as mTORC1 plays a major role in suppressing autophagy during the autophagy induction process (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009) whereas the activation of JNK during starvation leads to JNK-mediated Bcl-2 phosphorylation that subsequently releases Beclin 1 for the nucleation complex formation is mTOR-independent (Mehrpour et al., 2010; Wei et al., 2008).

Consistently, the JNK activation in autophagy deficient $Atg7^{-/-}$ MEFs seemed stronger than the $Atg7^{+/+}$ MEFs (Figure 4.11B and 4.11C). It is currently unknown if autophagy deficiency may induce higher JNK activation, as JNK is known to be activated during starvation to release Beclin 1 in promoting autophagy process (Wei et al., 2008). One possible explanation is, the ROS level in autophagy deficient cells may be higher and consequently, further promote JNK activation. Notably, it was previously reported that autophagy deficient plant have a higher oxidative stress level (Xiong et al., 2007). Moreover, the extent of JNK phosphorylation reduction seemed to be similar in both $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs when the Class III PI3K autophagy inhibitor, wortmannin (Arcaro and Wymann, 1993) was used (shown in Figure 4.10). Thus, these data indicate that JNK impairment signaling in $TSC2^{-/-}$ cells is autophagy-independent whereas the autophagy impairment (shown in Chapter 3) was largely attributed to the mTORC1 hyperactivity.

The oxidative stress-mediated JNK signaling is activated through a three-

tier cascade; initializing from upstream MAP3K such as ASK1 to MAP2K (MKK4, MKK7) and subsequently to MAPK (JNK). Notably, despite the significant JNK deficiency in $TSC2^{-/-}$ cells in response to oxidative stress, the upstream MAPK kinases including ASK1 and MKK4 were not perturbed in $TSC2^{-/-}$ cells (Figure 4.14).

Another profound observation that we have obtained from our studies was the significant reduction of tyrosine phosphorylation observed in TSC2-null cells (Figure 4.15A). During oxidative stress, tyrosine phosphorylation-dependent signal transduction takes place involving the large protein tyrosine phosphatase (PTP) superfamily, which comprises of these groups of enzymes: i) classical PTPs, ii) dual specificity PTPs and iii) low molecular weight PTPs (Andersen et al., 2001). Tyrosine phosphorylation plays a key role in the regulation of various processes such as cell to cell communication, cell transformation, regulation of gene transcription, mRNA processing, transportation of molecules in cells; in which it is implied in numerous cellular processes such as embryogenesis, tissue homeostasis, organ development and immunity. Meanwhile, tyrosine phosphorylation abnormalities have been associated with immune deficiency as well as in cancer (Alonso et al., 2004; Ullrich and Schlessinger, 1990).

The MKPs deactivate the MAPK signaling by dephosphorylating threonine and tyrosine at the TXY motif of the kinase activation loop (Davis, 2000). Elevated MKPs expression, such as MKP-1, have been shown to aid the oncogenic transformation by suppressing apoptotic consequences in response to oncogenic or genotoxic stress (Small et al., 2007; Wang et al., 2007a). In the

manifestations of TSC disease, benign tumours are formed in multiple organs (Gomez, 1991; van Slegtenhorst et al., 1997). In our study, we found that the expression of MKP-1 was higher in $TSC2^{-/-}$ cells (Figure 4.19). It is thus possible that in the absence of TSC, the MKP-1 expression is elevated and therefore, leads to tumours progression as seen in TSC disease-manifestated pathologies. Moreover, in agreement that JNK may also be dephosphorylated by dual-specificity MKPs (Karin and Gallagher, 2005), our data have also shown that TSC may regulate the MKPs (Figure 4.19 and 4.20).

Increased expression of MKP-1 is also found in patients with multiple sclerosis (Eljaschewitsch et al., 2006) and major depressive disorders (Duric et al., 2010). Moreover, elevated MKP-1 expression is also seen in certain cancers, such as in non-small cell lung cancer (Vicent et al., 2004) while high expression of MKP-1 confers drug resistance in melanoma as well as in glioblastoma (Kundu et al., 2010; Yu et al., 2012). Contradictorily, the loss of MKP-1 may also promote tumour metastasis and cell proliferation, such as seen in non-small cell lung cancer, prostate, colon, and bladder cancer (Loda et al., 1996; Tai et al., 2010). Moreover, MKP-1 induction is reduced in cells of severe asthmatics conditions (Bhavsar et al., 2008) and is essential for proper regulation of innate immunity (Zhang et al., 2009c). Therefore, MKP-1 seems to be a potential target for therapeutic purposes for various diseases, through the manipulation of its activity by using either specific inhibitors or inducers (Doddareddy et al., 2012).

Notably, in our study, we found that an interaction has existed between TSC2 and MKP-1 during oxidative stress. As shown in Figure 4.19, the mRNA

expression was higher in $TSC2^{-/-}$ cells treated with H_2O_2 whereas a drop of mRNA level occurred in $TSC2^{+/+}$ cells. Interestingly, more TSC2 protein was found to be bound to MKP-1 while a decreased of MKP-1 protein level was observed in oxidative stress-stimulated MEFs (Figure 4.20). These results possibly indicate that TSC suppresses MKP-1 both at the transcriptional level as well as the protein level, possibly by promoting its degradation. In agreement that MKP-1 is regulated in multiple contexts, earlier studies have shown that the transcriptional activity of MKP-1 is induced by various extracellular stimuli (Keyse, 1995, 1999), and while its stability is promoted through acetylation (Cao et al., 2008), the MKP-1 interaction with other MAPKs members is also able to enhance its catalytic activity (Hutter et al., 2000; Slack et al., 2001). Thus, these clearly indicate that TSC-mediated regulation of MKP-1 may involve these mechanisms.

On the other hand, MKP-1 protein has a half-life of about 1 hour and is targeted for degradation through the proteasome pathway for its inactivation (Brondello et al., 1999; Roth Flach and Bennett, 2010). Accordingly, as shown in Figure 4.16A, the addition of phosphatase inhibitors failed to further restore JNK activation in $TSC2^{+/+}$ cells, while a significant difference was observed in $TSC2^{-/-}$. These imply that the degradation of MKP-1 may be affected due to the absence of a functional TSC.

While it is well known that activation of JNK from ROS stimuli is linked to apoptosis (Hampton and Orrenius, 1998) as well as necrosis (Shen and Pervaiz, 2006; Vanden Berghe et al., 2010), we are interested to explore whether the

impaired JNK activation in $TSC2^{-/-}$ cells is functionally associated with ROS-mediated cell death. Interestingly, data from our study have revealed that the oxidative stress-mediated JNK activation had mainly promoted necroptotic cell death in $TSC2^{-/-}$ cells, as such cell death was effectively protected by Nec-1 (Figure 4.21 and 4.22). This suggests that the TSC-deficient cells may undergo necroptotic cell death during oxidative stress, although apoptotic cell death was also present, as indicated by the cleaved caspase 3 (Figure 4.22A).

In this study, we have also observed an insignificant difference of p38 activation in response to H_2O_2 in the TSC2 cells. Although it is generally known that MKP-1 may also regulate p38 (Roth Flach and Bennett, 2010), data from our study (Figure 4.14) has suggested that MKP-1 does not target p38 in our system. Meanwhile, we have also noted a downregulation of ERK and MKK4 (T261) phosphorylation status (Figure 4.14). It was previously reported that phosphatases such as protein phosphatase type 5 (PP5) could downregulate MKK4 (T261) independent of p38 and p-ERK (Zhou et al., 2004). As such, the regulation of other types of phosphatases by TSC1/TSC2 complex remains to be established.

Higher phosphatase activities may lead to other cognitive complications. Two recent studies have found that an elevated alkaline phosphatase activity is observed in Alzheimer's disease patients (Kellett et al., 2011; Vardy et al., 2012). Moreover, Protein Tyrosine Phosphatase Nonreceptor Type 5 was found to be involved in stress-mediated cognitive functions of psychopathology (Yang et al., 2012). Notably, TSC disease patients are also known to suffer from neurological disorders (Budde and Gaedeke, 2012). Thus, it would be interesting to know if the

deregulated phosphatase activity observed in this study may be accounted for the neurological disorders implicated in TSC disease.

Although it is not known on how exactly TSC mediates MKP-1 degradation, nevertheless data from our study strongly indicate that TSC is a key mediator of MKP-1 activity. It is also currently unknown if the elevated MKP-1 expression is associated with TSC-related pathologies, but as MKP-1 plays a diverse role in both the pathological and physiological functions (Doddareddy et al., 2012), this approach may have potential therapeutic implications in TSC-related pathologies. Collectively, our study has thus revealed a novel role of TSC in promoting stress-mediated JNK signaling via suppression of MKP-1, and its implications in cell death, as illustrated in Figure 4.23.

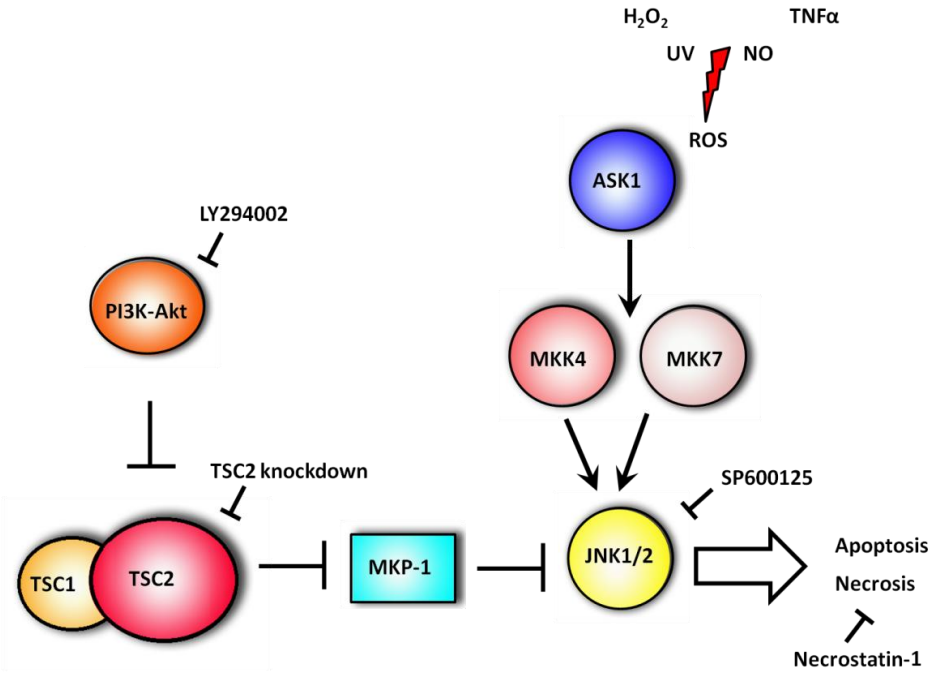

Figure 4.23 The regulation of TSC1/TSC2 complex in oxidative stress and cell death. In the presence of TSC protein, the MKP-1 activity is negatively regulated to activate JNK activity. Depending on the extent of stimuli, activated JNK may induce to apoptosis or necrosis. In TSC-null cells, the MKP-1 activity is high, leading to suppressed JNK activity and eventually necrotic cell death.

CHAPTER 5

GENERAL DISCUSSIONS AND CONCLUSIONS

Dysfunctional TSC, either due to its mutated *TSC1* or *TSC2* genes, leads to constitutive activation of mTORC1 that eventually causes numerous pathological conditions, including tumour formation in multiple organs, such as brain, kidneys, lung, heart and skin (Inoki and Guan, 2009). Presently, no effective systemic treatment is available for TSC-related disease (Budde and Gaedeke, 2012), in spite of the disastrous outcomes in TSC disease manifestations.

Meanwhile, autophagy is an evolutionary conserved mechanism in eukaryotes that maintains cytoplasmic homeostasis and enables cells to meet energy requirements under starvation, as well as to prolong survival under stress conditions (Rubinsztein et al., 2012). The mTORC1, a major cellular growth controller, is known to inhibit autophagy through inhibition of ULK1/2 complex and initiation of autophagy process (Rubinsztein et al., 2012). Hyperactivation of mTORC1 has always been linked to cancers and thus, extensive research has been performed on mTORC1 as a therapeutic target in cancer and several other diseases (Zoncu et al., 2011).

On the other hand, JNK is known to regulate cell death (apoptosis and/or necrosis) or cell survival, depending on the nature of the stimuli, the cellular extent and the periodic activation of JNK signaling (Weston and Davis, 2007). Among various stimuli, ROS and oxidative stress are particularly important for JNK activation (Torres, 2003). Notably, ROS, such as H_2O_2 has long been implicated in numerous diseases, including neurodegeneration, cancer, autoimmune diseases and ageing, DNA damage, lipids and proteins (Weinberg

and Chandel, 2009). Therefore, the role of JNK in mediating cell stress is indispensable in determining the final outcome of the cell's fate.

At present, the TSC-null cells are known to be more susceptible to various cell death stimuli, such as TNFα (Ghosh et al., 2006), glucose deprivation (Lee et al., 2007), and ER stress (Di Nardo et al., 2009; Kang et al., 2011). Despite the hypersensitivity of the TSC-null cells to various stimuli, the underlying mechanisms whether autophagy is involved has not been studied systematically.

Meanwhile, JNK is known to affect the TSC-mTORC1 signaling pathway via phosphorylation of raptor in a familial adenomatous polyposis model as well as during osmotic stress (Fujishita et al., 2011; Kwak et al., 2012). However, up to date, little is known whether the TSC-mTORC1 would affect JNK activation pathway under stress conditions.

Therefore, the main objective of this study is to investigate the regulatory roles of TSC in cellular stress response by focusing on autophagy, JNK signaling and cell death. By using multiple approaches, we have: (i) identified the role of TSC in autophagy under starvation conditions and (ii) revealed the role of TSC in JNK signaling in response to oxidative stress. Data from this study thus provided new insights in the functional roles of TSC in autophagy, JNK signaling and cell death regulation.

5.1 TSC-deficiency impairs autophagy and sensitizes cells to cell death

The TSC-null cells have been reported to be highly susceptible to various cell death stimuli due to various mechanisms, including increased p53 translation (Lee et al., 2007), increased activation of Rheb to ER stress (Kang et al., 2011),

lower NF-κB level (Ghosh et al., 2006), increased ER stress (Di Nardo et al., 2009; Ozcan et al., 2008), and defects in Akt activation (Huang et al., 2009). However, it is unknown if autophagy plays an important role in the sensitization of these cells to cell death. Therefore, in Chapter 3, we systematically investigated the role of autophagy in this process. Firstly, we compared the extent of cell death between the wild-type and $TSC1^{-/-}$ and $TSC2^{-/-}$ MEFs treated with various cell death stimuli (Figures 3.1, 3.2 and 3.3). We then examined the basal and inducible autophagy levels through autophagy flux studies, using starvation as an inducer of autophagy. Interestingly, the autophagy level in $TSC2^{-/-}$ cells was low, along with an elevated mTORC1 activity (Figures 3.4 and 3.5). Moreover, suppression of autophagy by chloroquine or Atg7 knockdown was found only effective in sensitizing cell death in the $TSC2^{+/+}$, but not the $TSC2^{-/-}$ cells (Figures 3.6 and 3.8), indicating that TSC2-deficient cells have an impaired autophagy, thus resulting these cells to be more susceptible to various cell death stimuli.

The notion that TSC2 deficiency causes defective autophagy, which has a pro-survival role, was further evidenced when $TSC2^{-/-}$ cells were rescued during autophagy activation through raptor knockdown and rapamycin (Figures 3.9 and 3.10). All these data clearly demonstrate that the pro-survival role of autophagy is important in cells that lack TSC.

Moreover, another remarkable observation was that supplementation of IGF-1 and leucine had different effects in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells (Figure 3.11 and 3.12). In general, nutrient supplementation may elicit two outcomes: 1) activates Akt pathway that thus suppresses apoptosis via multiple mechanisms

(Franke et al., 2003), or 2) blocks autophagy via activation of Akt-mTORC1 pathway and subsequently promotes cell death (He and Klionsky, 2009; Wu et al., 2009). The opposite outcome on cell death observed with nutrients supplementation between $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs could possibly be due to the different Akt status in both types of the cells. In $TSC2^{+/+}$ cells, nutrients supplementation rescues the cells from EBSS-induced starvation via activation of Akt; whereas in $TSC2^{-/-}$ cells, the Akt level is already low due to the negative feedback from activated S6K on IRS-1 (Harrington et al., 2004). On the other hand, in $TSC2^{-/-}$ MEFs, the activated Akt further increases mTORC1 activity thus leading to suppression of autophagy that finally enhances cell death.

Autophagy is well-known for its pro-survival role via recycling amino acid pool under starvation conditions (Mizushima, 2007). For example, *Atg3*, *Atg5*, and *Atg7* knockout mice undergo neonatal lethality due to deficiency in maintaining amino acid pool (Komatsu et al., 2005; Kuma et al., 2004; Sou et al., 2008). As autophagy is negatively regulated by mTORC1 through the phosphorylation of ULK1, ULK2 and Atg13 and a direct interaction of raptor to ULK1 (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009), whereas under starvation conditions, deactivated mTORC1 dissociates from this complexes that leads to the induction of autophagy (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009), it is understandable that the role of mTORC1 in mediating autophagy is indispensable. Moreover, as TSC1 and TSC2 proteins form a functional complex and integrate various upstream signals to represses mTORC1 (Laplante and Sabatini, 2012), it is thus intriguing to know

that TSC does play a key role in the regulation of autophagy in response to stress and starvation conditions. Therefore, data from our study clearly demonstrate that TSC is indeed an important factor that mediates mTORC1 and autophagy for cell survival when cells are undergoing stress conditions, such as starvation.

Our data are in fact, supported by several recent studies. For instance, the combination of treatments with mTORC1 and autophagy inhibition in TSC2-null xenograft tumours has led to synergised therapeutic efficacy (Parkhitko et al., 2011), thus suggesting that both autophagy and mTORC1 may be the therapeutic targets in TSC-related malignancy. In addition, another recent study has also implicated the role of TSC in autophagy and neurology (McMahon et al., 2012). Consistent with our results, they have showed that autophagy is suppressed in the brain tissues of TSC patients. They also observed seizures in *TSC1* and *PTEN* knockout mice, respectively, in which both have aberrant mTORC1 activation. Meanwhile, they also performed *Atg7* conditional deletion, in which seizures were promoted; thus indicating a link of TSC, mTORC1 and autophagy to epileptogenesis and neurology (McMahon et al., 2012).

It is important to note that since autophagy is a key mediator for cell survival, the importance of autophagy may be much implicated for the therapeutic benefits of TSC-related pathologies. For example, in TSC patients, the suppression of mTORC1 using mTOR inhibitors may cause the induction of autophagy, which may further enhance cell survival or tumour development as autophagy protects the cells from cell death induced by chemotherapeutic agents. In fact, current clinical trials for the treatment of TSC disease are targeting

mTOR-kinase inhibitors (Budde and Gaedeke, 2012). However, it has also been reported that the benign tumours seems to proliferate again when the administration of mTORC1-targeting drugs is stopped (Bissler et al., 2008). This may possibly indicate that the mTORC1-inhibitory drugs may activate the pro-survival role of autophagy that thus promotes the progression of tumour growth. Therefore, caution must be taken during the designation of treatments for TSC- or mTOR-related diseases.

Taken together, we provide evidence demonstrating a novel function of TSC in control of cell death in response to nutritional stress/starvation via suppression of mTORC1 and promotion of autophagy. Cells deficient of TSC are thus susceptible to cell death due to impaired autophagy, which is known to be an important pro-survival mechanisms against stress-mediated cell death.

5.2 TSC promotes JNK activation via downregulation of tyrosine phosphatase

At present, there is evidence indicating the crosstalk between mTORC1 and JNK signaling pathway. On one hand, JNK is known to phosphorylate and negatively regulation the function of IRS-1, which lies upstream of Akt-mTORC1 pathway (Aguirre et al., 2000; Hilder et al., 2003; Lee et al., 2003). On the other hand, JNK is involved in phosphorylating raptor to promote mTORC1 activity in intestinal tumour cells (Fujishita et al., 2011) and during osmotic stress (Kwak et al., 2012). However, to the best of our knowledge, so far there is no clear evidence whether the TSC-mTORC1 pathway has any regulatory effect on JNK signaling in response to oxidative stress. Therefore, in Chapter 4, we have

expanded the scope of TSC function and examined the role of TSC in oxidative stress-mediated JNK signaling and cell death.

One key finding from this part of our study is impairment of JNK signaling was observed in TSC-null cells induced with various stress stimuli (Figures 4.1-4.4). We first found that the deficient JNK activation in $TSC2^{-/-}$ MEFs in response to oxidative stress was not associated with the hyperactivity of mTORC1, low autophagy, or impairment of the upstream JNK signaling (Figure 4.9-4.14). It is known that mTORC1 suppresses autophagy through the phosphorylation of ULK1 complex (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). On the other hand, the autophagy signaling involving JNK-mediated Bcl-2 phosphorylation seems to be mTORC1-independent (Mehrpour et al., 2010; Wei et al., 2008). Thus, it is understood that the impairment of JNK regulation in $TSC2^{-/-}$ MEFs was not associated with its autophagy deficiency.

Remarkably, we found that $TSC2^{-/-}$ MEFs had a significantly reduced tyrosine phosphorylation in response to H_2O_2 (Figure 4.15A), and a general tyrosine phosphatase inhibitor, sodium orthovanate was able to restore JNK activation (Figure 4.16). In fact, tyrosine phosphorylation is a key signaling event that plays a critical role in many physiological processes such as embryogenesis, tissue homeostasis, organ development and immunity, whereas abnormalities of tyrosine phosphorylation have been associated with immune deficiency and cancer (Alonso et al., 2004; Ullrich and Schlessinger, 1990). Thus, it remains to be further investigated whether the impaired JNK activation and the reduced tyrosine phosphorylation is causatively associated with the pathology manifested

in in TSC disease.

In search of the molecular mechanisms underlying the impaired JNK activation in $TSC2^{-/-}$ MEFs, we first excluded the possibility that it is caused by the deficiency in the activation of upstream kinases such as ASK1 and MKK4 (Figure 4.14). We then attempted to establish the role of protein phosphatases. MKP-1 is one of the key phosphatases in the regulation of JNK signaling (Hirsch and Stork, 1997; Kamata et al., 2005). One important finding is that there is a significantly higher mRNA level of MKP-1 in $TSC2^{-/-}$ MEFs comparing to the $TSC2^{+/+}$ MEFs (Figure 4.19). Interestingly, from the protein-protein interaction study, MKP-1 was found to bind to TSC2, with an increased binding of TSC2 and a decreased level of MKP-1 protein level were observed in H_2O_2-treated $TSC2^{+/+}$ MEFs (Figure 4.20). These data thus indicate the possibility that TSC might mediate the MKP-1 function via transcriptional down-regulation and through protein-protein interaction to mediate its degradation. In the absence of TSC, these functions are probably perturbed, thus leading to enhanced MKP-1 activity and subsequently causes impaired JNK activation. However, the underlying mechanism on how TSC regulates MKP-1 needs to be further elucidated.

Data from this study have thus indicated that TSC plays an additional role which is independent of the mTORC1 regulation, in mediating MKP-1 activity. At present, MKP-1 serves as a potential target for therapeutic therapies as it plays a diverse role in both physiological and pathological processes (Doddareddy et al., 2012). Elevated MKP-1 expression is linked to drug resistance in melanoma, glioblastoma and breast cancer (Kundu et al., 2010; Small et al., 2007; Yu et al.,

2012), while its overexpression is seen in non-small cell lung cancer (Vicent et al., 2004). Although there are also reported studies that the loss of MKP-1 may promote metastasis and cell proliferation in non-small cell lung cancer, prostate, colon, and bladder cancer (Loda et al., 1996; Tai et al., 2010), nevertheless, MKP-1 appears to be an important therapeutic target in these cancers (Doddareddy et al., 2012). Therefore, data from this study provide useful insights for establishing the role of MKP-1 in TSC-related pathologies.

5.3 Conclusions

In this thesis, we have systematically investigated the roles of TSC in mediating stress-mediated cellular responses by focusing on autophagy, JNK and cell death. This study consisted of mainly two parts: (i) investigation on the role of TSC in regulation of autophagy and cell death under starvation and, (ii) examination on the role of TSC in JNK signaling and cell death under oxidative stress. The main findings are:

1. Impaired autophagy due to constitutive mTOR activation sensitizes TSC2-null cells to cell death under stress
    i. $TSC2^{-/-}$ cells have lower basal and inducible autophagy level due to constitutive mTORC1 activation
    ii. Suppression of autophagy failed to sensitize $TSC2^{-/-}$ cells to cell death due to low autophagy level
    iii. Activation of autophagy with starvation in $TSC2^{-/-}$ cells protected cells from death
    iv. Nutrients supplementation protects against cell death in

$TSC2^{+/+}$cells, but enhances cell death in $TSC2^{-/-}$ cells under starvation condition

2. TSC promotes JNK activation via downregulation of MKP-1
    i. Activation of JNK signaling in response to ROS and other stimuli is impaired in $TSC2^{-/-}$ MEFs
    ii. $TSC2^{-/-}$ cells have a significantly lower tyrosine phosphorylation activity
    iii. TSC binds to MKP-1 and regulates its expression
    iv. JNK impairment sensitizes $TSC2^{-/-}$ cells to necrosis

Taken together, we have demonstrated the crucial role of TSC in autophagy, JNK signaling and cell death regulation. The main findings from this study are illustrated in Figure 5.1. We believe that data from this study will contribute to the current literature for the function of TSC, particularly in control of autophagy, JNK activation and cell death, which provide a foundation for development of novel therapeutic strategies for TSC-related pathologies.

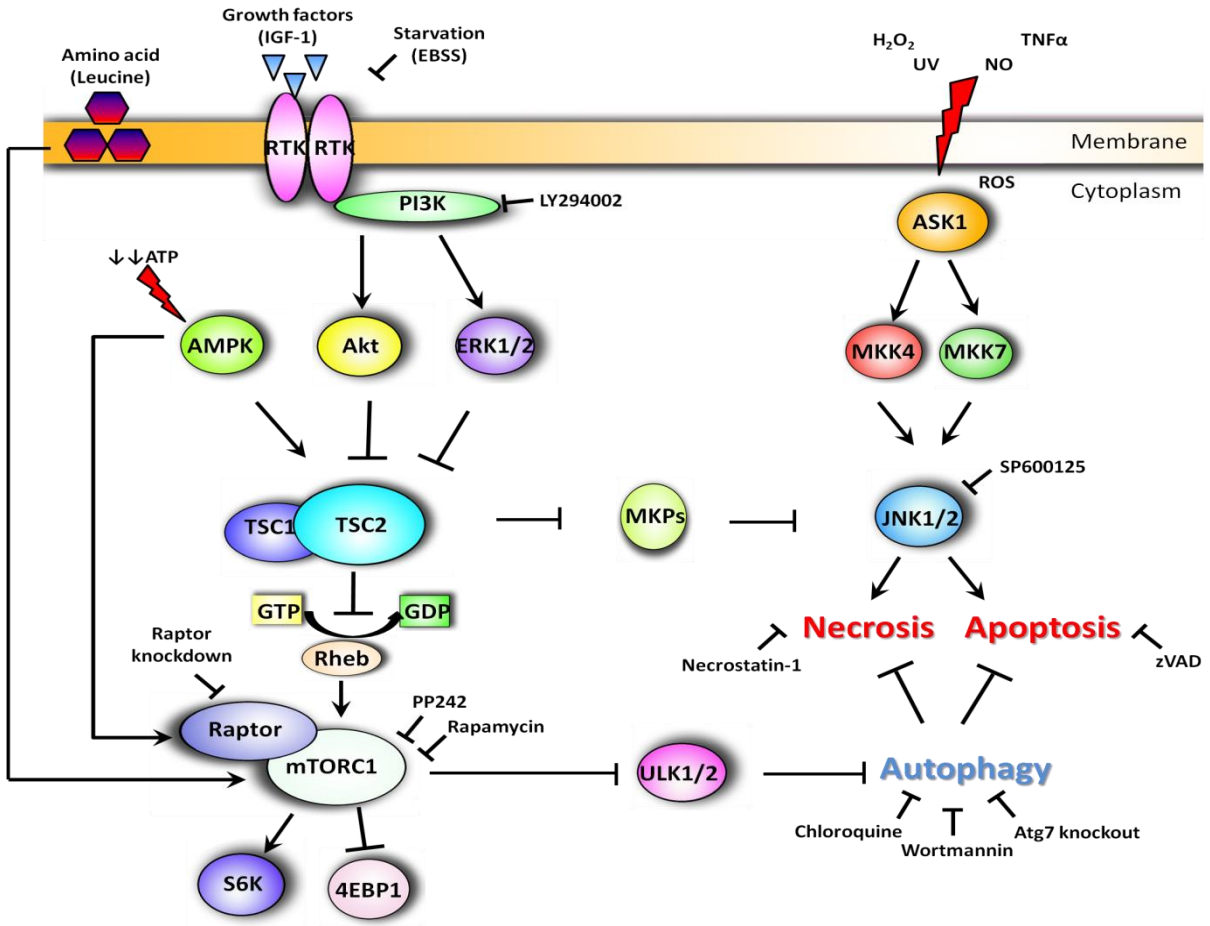

Figure 5.1 TSC as a key stress modulator in control of mTORC1, JNK, autophagy and cell fate in response to various cell stress stimuli.

CHAPTER 6

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